Interactions between *Neoparamoeba* spp. and Atlantic salmon (*Salmo salar* L.) immune system components

By

Kally Anne Gross

(B.Biomed.Sci.Grad.Dip App.Sci.B.App.Sci Hons)

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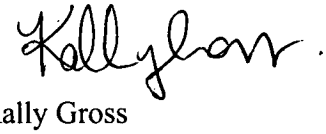

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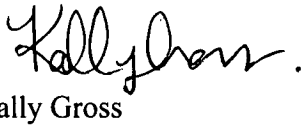

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Abstract

The protozoan parasite *Neoparamoeba* spp. infects the gills of marine cultured Atlantic salmon in Tasmania, Australia causing amoebic gill disease (AGD) and if untreated causes significant production losses. Knowledge of the interaction between *Neoparamoeba* spp. and host immune factors or cells is limited; specific anti-*Neoparamoeba* spp. antibodies are present in the serum of fish naturally exposed and sheep anti-*Neoparamoeba* spp. antibodies do not affect the viability of *Neoparamoeba* spp. This thesis investigates *in vitro* and *in vivo* interactions between the host (Atlantic salmon) and *Neoparamoeba* spp. The effect of *Neoparamoeba* spp. on *in vitro* innate immune cell function was investigated. Sonicated *Neoparamoeba* spp. did not stimulate a respiratory burst response from anterior kidney leukocytes ($P > 0.05$). However, there was evidence of priming as cells incubated with the pathogen and then stimulated with phorbol myristate acetate (PMA) produced more ($P < 0.05$) reactive oxygen species (ROS) than cells stimulated with PMA alone. *Neoparamoeba* spp. culture supernatant did not affect immune cell function ($P > 0.05$). Further *in vitro* investigations found that there was no effect of host serum and mucus on parasite viability ($P > 0.05$). The effect of *Neoparamoeba* spp. infection on *in vitro* gill cell function was assessed. A technique was developed to isolate viable gill cells from perfused gills. Gill cells were tested for their ability to produce ROS, engulf yeast cells and chemotaxis. Cells did not produce ROS in the presence of PMA above basal levels, did not chemotactically migrate and few cells were capable of phagocytosis despite cell viability greater than 80 %. In light of these observations further studies utilised anterior kidney cells. Anterior kidney cells were used to investigate the effect of gill infection on innate immune cell function

(ROS production, phagocytosis and chemotactic ability) *ex vivo*. There was a significant ($P < 0.05$) effect of infection on ROS production 8 and 11 days post *Neoparamoeba* spp. exposure. A previous study reported increased resistance to AGD in fish that had been infected with *Neoparamoeba* spp.. However, these results were not consistent with the experiences of commercial aquaculture ventures that have performed similar investigations; therefore the study was repeated. It was found that recovered fish were equally susceptible ($P > 0.05$) to disease upon re-infection as naïve fish. Immunohistochemistry was used to identify Ig bearing cells in the gills of Atlantic salmon. The spatial and temporal distribution of Ig bearing cells was not different between affected and un-affected fish ($P > 0.05$). Together the data presented in this thesis identify that the interactions between host and pathogen are complex. The parasite may elude destructive immune cell defences by avoiding initiation of deleterious mechanisms or by immunosuppression.

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Last, but certainly not least, I would like to thank my family and friends. I never could have made it to the point of submission without the support, encouragement and love that you all gave me, all of the time. Thank you.

List of abbreviations

α2-M	alpha-2 macroglobulin
AB/PAS	Alcian blue/periodic acid Schiff stain
ASC	Antibody secreting cell
AGD	Amoebic gill disease
AKCs	Anterior kidney cells
ANOVA	Analysis of variance
APR	Acute phase response
BCA	Bovine calf albumin
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
Con A	Concavalin A
CpG	Cytosine-phosphodiester-guanine oligodeoxynucleotides
CPE	Cytopathic effect
CVS	Central venous sinus
DAB	Diaminobenzidine
DN	Deoxyribonuclease
DPE	Days post *Neoparamoeba* spp.-exposure
DTT	Dithiothreitol
ECP	Extracellular product
ECS	*Escherichia coli* culture supernatant
EDTA	Ethlyene diaminetetra acetic acid
EGC	Eosinophilic granular cell
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N'-tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
EST	Expressed sequence tag
FCS	Fetal calf serum
FMC	Fully migrated cell
FOV	Field of view
FW	Freshwater
GCs	Gill cells
H & E	Haematoxylin and eosin stain
HBSS	Hank's balanced salt solution
HEWL	Hen egg white lysozyme
HIER	Heat induced epitope retrieval
HRP	Horseradish peroxidase
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL-1β	Interleukin 1β
IL-4	Interleukin-4
IL-8	Interleukin 8
IL-10	Interleukin-10
IL-12	Interleukin-12
ILV	Intralamellar vesicle
iNOS	Inducible nitric oxide synthase
kDa	Kilodalton
L15	Leibovitz's media
LN	Natural log
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MC	Migrating cell
MHC	Major histocompatibility complex
MHC-II	Major histocompatibility complex class II molecule
Mn	Melanomacrophage
MN	Migrated neutrophil
MM	Migrated macrophage

mRNA	Messenger ribonucleic acid
MST	Mean survival time
mw	Molecular weight
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
Nspp	*Neoparamoeba* spp.
OD	Optical density
P	Phagocyte
P/S	Penicillin and streptomycin
PBL	Peripheral blood leukocyte
PBS	Phosphate buffered saline
PI	Phagocytic index
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PR	Phagocytic rate
qRT-PCR	Quantitative real time polymerase chain reaction
RBC	Red blood cell
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RNI	Reactive nitrogen intermediates
RPS	Relative percent survival
RT	Room temperature
SAA	Serum amyloid A
SAP	Serum amyloid P
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Standard error
SEM	Standard error of mean
SM	Low fat skimmed milk powder
SOD	Superoxide dismutase
SW	Seawater
SWC	Seawater Cortland's media
SWD	Seawater Davidson's fixative
SWF	Seawater formalin fixative
TBS	Tris buffered saline
TEMED	N,N,N',N'-Tetramethy lethylenediamine
TNF-α	Tumor necrosis factor-alpha
WBC	White blood cell

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Chapter 1 General Introduction

Parasitic infections of fish

Parasites often cause little damage to fish in their natural habitat however their presence in the aquaculture environment may result in disease, pathological changes, decreased condition and/or reduction in market value of the cultured animal (Dickerson & Clark 1998; Scholz 1999; Kent 2000; Jones 2001; Buchmann & Lindenstrom 2002). Mortality or morbidity of parasitised fish may occur due to osmoregulatory disturbances (Grimnes & Jakobsen 1996), pathological changes (Dezfuli, Pironi, Giari, Domeneghini & Bosi 2002), immunosuppression (Scharsack, Steinhagen, Kleczka, Schmidt, Korting, Michael, Leibold & Schuberth 2003), secondary infections (Mustafa, Speare, Daley, Conboy & Burka 2000) or stress (Bowers, Mustafa, Speare, Conboy, Brimacombe, Sims & Burka 2000).

Arguably the first step in creating a prophylactic treatment for parasitic diseases is to understand the host-parasite interactions on an immunological level. To do this one must investigate the effect of the parasite on the host immune components *in vitro*, *in vivo* and *ex vivo* and *vice versa*. This thesis is concerned with understanding the interactions that occur between the protozoan parasite *Neoparamoeba* spp. (Figure 1-1) and immune system components of Atlantic salmon (*Salmo salar*) during amoebic gill disease (AGD).

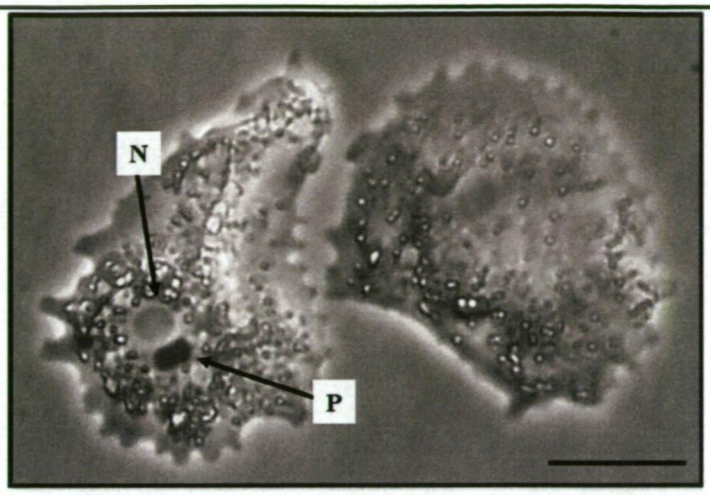

Figure 1-1 Plastic adhered *Neoparamoeba* spp. isolated from the gills of AGD-affected Atlantic salmon (*Salmo salar* L.), note nucleus (N) and parasome (P) (photo from Morrison, Crosbie, Cook, Adams & Nowak 2005). Bar = 20 µm.

A brief history of Atlantic salmon aquaculture and AGD in Tasmania

Atlantic salmon aquaculture production in Tasmania, Australia began in 1984/85 and the first harvest was 53 tonnes in the summer of 1986/87 (Dix 1986; Munday, Foster, Roubal & Lester 1990). The onset of significant (up to 50%) mortalities of sea-cage reared Atlantic salmon occurred soon after the introduction of Atlantic salmon smolt to the marine environment. Affected fish had severe mucoid branchialitis (Munday 1986). No definitive diagnosis was made however large numbers of amoeboid protozoans were identified in wet preparations made from the gills of affected fish. It was not until the summer of 1987/88 that the disease causing organism was presumptively identified as the free-living protozoan parasite *Paramoeba pemaquidensis* (Roubal, Lester & Foster 1989). At that time *P. pemaquidensis* was considered to be the aetiological agent of amoebic gill disease (AGD) in coho salmon (*Oncorhynchus kisutch*) in Washington, USA (Kent, Sawyer & Hedrick 1988). The parasite was re-classified and subsequently transferred to the genus *Neoparamoeba* Page, 1987.

After the initial deaths of cultured Atlantic salmon to AGD, the disease was controlled by freshwater bathing (Foster & Percival 1988), a mitigation process which is still used on farms today (Roberts & Powell 2003). More recently, *N. branchiphila* have been isolated from the gills of AGD-affected fish (Dyková, Nowak, Crosbie, Fiala, Pecková, Adams, Macháèková & Dvoráková 2005).

Throughout this thesis the causative agent of AGD may either be referred to as *N. pemaquidensis*, *Neoparamoeba* sp. or *Neoparamoeba* spp. dependent upon the method used to identify the organism. Furthermore, two of the chapters (chapter 6 & 7) were published prior to the discovery of *N. branchiphila* and *Neoparamoeba* sp. or *N. pemaquidensis* is used in reference to the causative agent. However, irrespective of the term used to describe the organism, it is referring to the species of *Neoparamoeba* which are the aetiological agent of AGD. This may also include *N. aesturina* as its role in the aetiology of AGD is yet to be disproved (Dyková *et al.* 2005).

Neoparamoeba spp. infect only the gills (Dyková, Figueras & Novoa 1995; Zilberg & Munday 2000) of fish cultured in the marine environment. AGD-associated histopathology has not been found in wild-populations of fish around AGD-affected farms or fish pens (Douglas-Helders, Dawson, Carson & Nowak 2002; Nowak, Dawson, Basson, Deveney & Powell 2004). *Neoparamoeba* spp. is regarded by some to be the amoebic parasite of most economic concern to the aquaculture industry (Munday, Zilberg & Findlay 2001). Globally, AGD has been diagnosed in a number of countries affecting many marine fish species. Countries in which AGD-affected fish have been identified include; Ireland,

France, Chile, Spain, USA and New Zealand. Fish species aside from Atlantic salmon that are affected by AGD include rainbow trout (*Oncorhynchus mykiss*), coho salmon, chinook salmon (*Oncorhynchus tshawytscha*), turbot (*Scophthalmus maximus*) and sea bass (*Dicentrarchus labrax*) (Kent *et al.* 1988; Munday *et al.* 1990; Dyková *et al.* 1995; Rodger & Mc Ardle 1996; Findlay & Munday 1998; Clark & Nowak 1999; Dyková, Figueras & Peric 2000; Munday *et al.* 2001).

Grossly, AGD is characterised by white, raised mucoid patches on the gills (Figure 1-2) which have close conformity to the microscopic presentation of the disease (Figure 1-3) (Adams, Ellard & Nowak 2004). That is, mucoid patches can be "matched" to the lesion presence on the gill, providing no other gill condition is present (Adams *et al.* 2004). Although it is clear a precursor for lesion development is attachment of the trophozoites to the gill epithelium (Zilberg & Munday 2000; Adams & Nowak 2003; Adams & Nowak 2004a; Adams & Nowak 2004b) the mechanism of attachment by *Neoparamoeba* spp. trophozoites to the gill epithelium has not been elucidated.

Figure 1-2 Characteristic white, mucoid patch (MP) associated with amoebic gill disease (Photo courtesy of Dr. Mark Adams).

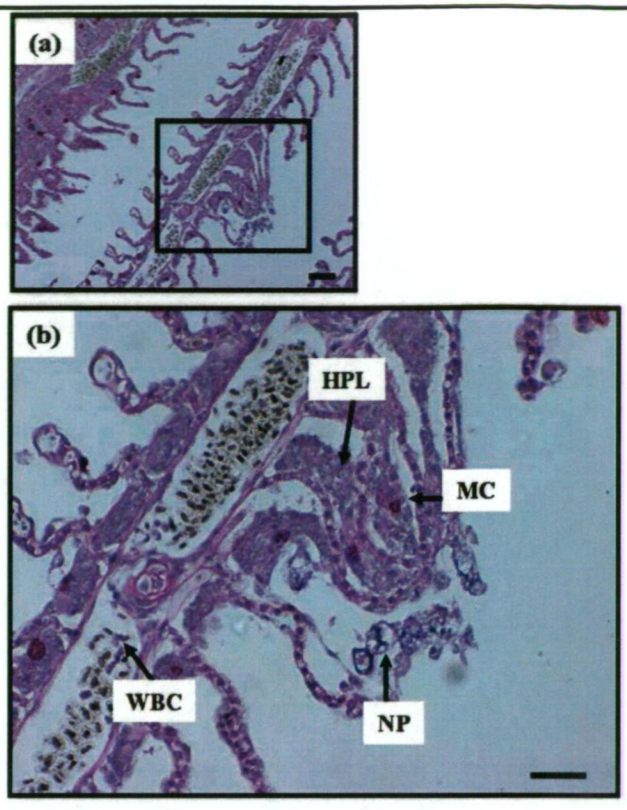

Figure 1-3 Histological sections of gill stained with alcian blue/periodic acid Schiff (AB/PAS). (b) = insert from (a) at higher magnification (× 400). Mucous cells (MC) stain bright pink, amoebae trophozoite (NP) stain bright blue and the rest of the tissue stains purple. Peripheral blood cells including white blood cells (WBC) may be seen in the central venous sinus, hyperplastic lesions (HPL) can be seen as epithelial cells of the lamellae fusing together. Bars = 50 μm.

Protective immune responses of fish against parasites

The immune response of Atlantic salmon affected with AGD is relatively undescribed compared to other parasitic diseases of fish. The presence of anti-*Neoparamoeba* spp. antibodies in the serum of Atlantic salmon exposed to *Neoparamoeba* spp. through infection or immunisation has been demonstrated (Findlay, Helders, Munday & Gurney 1995; Akhlaghi, Munday, Rough & Whittington 1996; Findlay & Munday 1998; Zilberg & Munday 2000; Gross, Carson & Nowak 2004; Vincent, Morrison & Nowak 2006). However the antibodies did not confer protection as AGD persisted in these populations of fish. Gill infection with *Neoparamoeba* spp. is responsible for alterations in immune-

regulatory gene expression in the anterior kidney and liver (Bridle, Morrison, Cupit Cunningham & Nowak 2006a; Bridle, Morrison & Nowak 2006b) indicating that this localized gill infection is capable of altering systemic immune responses.

Whilst there are many similarities between mammalian and teleost immune responses, direct comparisons could be misleading. Arguably the most fundamental reason for the sole focus on fish is that teleost fish are poikilotherms and humans are endotherms. Both innate and specific immune responses in poikilotherms are temperature-dependent (Rijkers, Frederix-Wolters & van Muiswinkel 1980) and therefore may differ to those in endotherms. In addition, protistans such as *Giardia* spp. and *Entamoeba* spp. that cause disease in humans are endoparasites and *Neoparamoeba* spp. is an ectoparasite. The results in this thesis are discussed in relation to teleost immune responses with occasional references to human immune responses only when there are direct similarities or little evidence from the literature regarding the teleost immune system.

Cellular mechanisms of parasitic control in fish

During ectoparasitic infections it is essential that the cellular (Nakayasu, Tsutsumi, Oseko & Hasegawa 2005) and humoral (Noga, Fan & Silphaduang 2002) components of the fish immune system have direct contact with the parasite in order to resolve infection. During the progression of AGD, localized epithelial changes such as epithelial oedema, epithelial cell proliferation and desquamation at the point of trophozoite attachment have been reported (Adams & Nowak 2004a). Leucocytic infiltrations in the central venous sinus, filamental connective tissue, filamental vascular tissue and within areas of hyperplastic epithelium are

also characteristic of the disease (Roubal *et al.* 1989; Munday *et al.* 1990; Adams & Nowak 2001; Adams & Nowak 2003; Adams *et al.* 2004; Adams & Nowak 2004a; Adams & Nowak 2004b).

Identification of specific leucocytic sub-populations in the gill of AGD-affected fish has been limited to the immunohistochemical recognition of major histocompatibility class II (MHC-II) bearing cells (Morrison, Koppang, Hordvik & Nowak 2005) and presumptive morphological characterisation of macrophages, lymphocytes, eosinophilic granular cells (EGCs) and neutrophils (Adams & Nowak 2001; Bridle, Butler & Nowak 2003; Adams & Nowak 2004b). In other parasitic gill diseases of fish, immunohistochemistry has been used to identify infiltrations of EGCs (Roberts, Johnson & Casten 2004), rodlet cells (Dezfuli, Giari, Konecny, Jaeger & Manera 2003) and macrophages (Dezfuli, Giari, Simoni, Shinn & Bosi 2004).

As *Neoparamoeba* spp. attach exclusively to the gill (Dyková *et al.* 1995; Zilberg & Munday 2000) and cells of immunological lineage have been identified in the gill (Lumsden, Ostland, Byrne & Ferguson 1993; Moore, Ototake & Nakanishi 1998) it is hypothesised that gill cells may be involved in immunological control of *Neoparamoeba* spp.. The gill plays an important role in vaccine uptake (Moore *et al.* 1998) and immunoglobulin production (Lumsden *et al.* 1993; Saha, Suetake & Suzuki 2004). Plasma cells have been identified in the gills of vaccinated fish (Grontvedt & Espelid 2004) and antigen processing capabilities of

gill cells have been demonstrated (Torroba, Anderson, Dixon, Casares, Varas, Alonso, Delmoral & Zapata 1993).

The ability of immune cells to phagocytose, migrate to the site of infection and produce reactive oxygen species (ROS) are actions which play a significant role in pathogen destruction or control by the fish immune response (Bower & Evelyn 1988; Bayne & Gerwick 2001; Ellis 2001; Jones 2001; Fast, Sims, Burka, Mustafa & Ross 2002b; Magnadottir 2006; Sitja-Bobadilla, Redondo, Bermudez, Palenzuela, Ferreiro, Riaza, Quiroga, Nieto & Alvarez Pellitero 2006). Phagocytosis is initiated by receptor-mediated endocytosis or non-specific hydrophobic interactions of the cell membrane with target particles (Allen & Stevens 1992; Secombes & Fletcher 1992; Ellis 1999; Neumann, Stafford, Barreda, Ainsworth & Belosevic 2001). Many morphologically different cell types possess phagocytic capabilities including immune (Secombes & Fletcher 1992) and non-immune cells (Glenney & Petrie-Hanson 2006).

Many studies have reported that leucocytes at the site of inflammation are more active with respect to respiratory burst and phagocytosis than leucocytes from areas distal to the inflammation (Park & Wakabayashi 1992; Itou, Iida & Kawatsu 1996; Matsuyama & Iida 1999; Matsuyama, Iida & Kurokura 1999; Kodama, Tijiwa, Moritomo & Nakanishi 2002; Serada, Moritomo, Teshirogi, Itou, Shibashi, Inoue & Nakanishi 2005). For example, neutrophils isolated from the swim bladder of carp (*Cyprinus carpio*) and tilapia (*Oreochromis niloticus*) that

had been injected with *Escherichia coli*, had significantly higher phagocytic activity than peripheral blood neutrophils (Matsuyama & Iida 1999).

Alterations in phagocytic rate (the proportion of phagocytes actively phagocytosing) and phagocytic index (the mean number of particles each phagocyte has ingested) often occur during parasitic infections in fish and results may be used to understand the host immunological response to the infection (Leiro, Arranz, Iglesias, Ubeira & Sanmartin 2004; Chaves, Luvizzotto-Santos, Sampaio, Bianchini & Martinez 2006). The phagocytic rate and index of anterior kidney leucocytes isolated from pompano (*Trachinotus marginatus*) naturally and experimentally infested with the gill ectoparasite *Bicotylophora trachinoti* was increased 15 days post-exposure (DPE) and decreased 30 DPE compared to unexposed fish (Chaves *et al.* 2006). There is *in vivo* evidence of host gill cells such as macrophages interacting directly with *Neoparamoeba* spp. (Adams & Nowak 2003) and phagocytic cells clearing amoebic debris from within interlamellar cysts (Adams & Nowak 2001).

One of the most important anti-microbial functions of phagocytes is the increase in oxygen consumption that can occur during phagocytosis, this is termed the "respiratory burst", which corresponds to the production of reactive oxygen intermediates (ROI) or ROS (Chanock, Benna, Smith & Babior 1994; Babior 1999). During phagocytosis the production of ROS occurs exclusively within the phagolysosome, however if the respiratory burst is activated by means other than

phagocytosis for example adherence to non-phagocytosable substrates such as opsonized surfaces, the ROS are released from the cell into the surrounding environment (Chanock *et al.* 1994; Babior 1999).

In mammals the enzyme responsible for the respiratory burst response is termed either respiratory burst oxidase (Chanock *et al.* 1994) or NADPH oxidase (reduced nicotinamide adenine dinucleotide phosphate) (Elbim, Guichard, Dang, Flay, Pedruzzi, Demur, Pouzet, El Benna & Gougerot-Pocidalo 2005). The production of ROS by fish phagocytes has been demonstrated and it appears that similar mechanisms of activation occur in fish phagocytes as do in mammalian cells, including the presence of NADPH oxidase-like components (Secombes, Cross, Sharp & Garcia 1992; Neumann *et al.* 2001), however pathways are not fully characterised and therefore the mammalian ROS production system is discussed.

NADPH oxidase catalyses the reduction of oxygen to O_2^- at the expense of NADPH and is dormant in resting cells (Chanock *et al.* 1994). The NADPH oxidase complex consists of a number of components residing throughout the cytosol and membrane of the cells (Babior 1999). NADPH oxidase is a multi-component enzyme composed of $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, GTPase *rac*2 and cytochrome b558 (comprised of $p22^{phox}$ and $gp91^{phox}$) (DeLeo, Renee, McCormick, Nakamura, Apicella, Weiss & Nauseef 1998; Babior 1999; Dang,

Dewas, Gaudry, Fay, Pedruzzi, Gougerot-Pocidalo & El Benna 1999; Dewas, Dang, Gougerot-Pocidalo & El Benna 2003; Elbim *et al.* 2005).

All 5 NADPH oxidase subunit cDNAs have been sequenced from Japanese pufferfish (*Takifugu rubripes*) (Inoue, Suenaga, Yoshiura, Moritomo, Ototake & Nakanishi 2004). Based upon reported expressed sequence tag (EST) sequences, NADPH oxidase complex-like components have also been found in European flounder (*Platichthys flesus*) (DV569895[¤]), Chinese perch (*Siniperca chuatsi*) (CO036795[¤]), Atlantic cod (*Gadus morhua*) (CO541629[¤]), channel catfish (*Ictalurus punctatus*) (CK417836[¤]) and zebrafish (*Danio rerio*) (AW419843[¤]). Together, the identification of those sequences suggests that the production of super oxide anion by phagocytes from fish is mediated by similar mechanisms as phagocytes from mammals.

ROS production by fish leucocytes can be affected by *in vitro* or *ex vivo* exposure to parasites and may identify the mechanism by which the parasite interacts with the host. ROS produced by rainbow trout anterior kidney leucocytes following *in vitro* exposure to *G. derjavini* was elevated compared to unexposed leucocytes (Buchmann & Bresciani 1999). Conversely, when anterior kidney leucocytes isolated from the olive flounder (*Paralichthys olivaceus*) were incubated with viable *Uronema marinum*, production of ROS was inhibited (Kwon, Kim, Chung, Lee & Kim 2002). The production of ROS by anterior kidney leucocytes isolated

[¤] GenBank accession number. www.ncbi.nlm.nih.gov

from turbot infected with *Tetramicra brevifilum* is less than that produced by leucocytes isolated from uninfected fish (Leiro, Iglesias, Parama, Sanmartin & Ubeira 2001) suggesting an immunosuppressive effect of the parasite on the host. Expression of $p40^{phox}$ in the gill of Atlantic salmon affected with AGD was down-regulated compared to expression levels from unaffected fish (Morrison, Cooper, Koop, Rise, Bridle, Adams & Nowak 2006). Whether the expression level of $p40^{phox}$ in the gill translates to decreased ROS production by gill cells or anterior kidney cells is unknown.

Humoral mechanisms of parasitic control in fish

Humoral components of the fish innate immune system include; anti-bacterial peptides, proteases, complement, lysozyme and plasma proteins (reviewed by Alexander & Ingram 1992; Buchmann 1999; Bayne & Gerwick 2001; Ellis 2001; Magnadottir 2006). Of the innate humoral components complement is the most effective at killing parasites. The complete complement cascade has not been completely characterised in fish, however it appears that the same proteins are involved in fish as in mammals (Sakai 1992; Nonaka & Smith 2000; Smith, Clow & Terwilliger 2001; Holland & Lambris 2002; Boshra, Li & Sunyer 2006). In humans the complement system is composed of over 35 individual proteins (reviewed by Carroll 1998). Activation of complement mediates chemotaxis, phagocytosis and respiratory burst production by host leucocytes and also direct lysis of pathogens (Weir & Stewart 1997).

The fish complement cascade can be activated via three pathways; classical (antibody mediated), alternative (direct contact between target and C3 convertase) and lectin mediated. All three pathways merge at the critical complement component 3 (C3). Ultimately resulting in the production of the membrane attack complex (MAC) and lysis of the target organism (Nakao & Yano 1998; Nonaka & Smith 2000; Holland & Lambris 2002; Boshra *et al.* 2006). Complement from teleosts has been implicated in the killing of fish parasites such as *Gyrodactylus salaris* (Harris, Soleng & Bakke 1998) and *Cryptobia salmositica* (Forward & Woo 1996) and may be a critical component of host resistance to invading pathogens.

The enzyme lysozyme is most effective at killing Gram-positive bacteria due to its action on the polysaccharide cell wall (reviewed by Ellis 1999). Serum and mucus lysozyme levels may be elevated in viral or bacterial diseases (reviewed by Ellis 1999; Ellis 2001) and a role in the protection of fish against parasites has also been suggested (Buchmann 1999; Ross, Firth, Wang, Burka & Johnson 2000; Fast, Ross, Mustafa, Sims, Johnson, Conboy, Speare, Johnson & Burka 2002a; Fast *et al.* 2002b). For example; Atlantic salmon, which are more susceptible to *Lepeophtheirus salmonis* (sea louse) have much lower levels of mucous lysozyme and proteases compared to less susceptible coho salmon and rainbow trout (Fast *et al.* 2002b).

Parasitism can stimulate high levels of mucus production as a protective response of the host (Buchmann 1999; Ross *et al.* 2000; Jones 2001; Bosi, Arrighi, Di Giancamillo & Domeneghini 2005). Whether gill mucus plays a role in controlling *Neoparamoeba* spp. infection is unknown, however increases in gill mucous cell density have been reported in fish affected by AGD (Nowak & Munday 1994; Zilberg & Munday 2000; Adams & Nowak 2003; Roberts & Powell 2003). Furthermore regions of the gill which are rich in mucous cells have fewer attached trophozoites (Adams & Nowak 2003; Adams & Nowak 2004b).

The protein composition of plasma in fish is altered by pro-inflammatory cytokine production during the very early stages of the acute inflammatory response (Bayne & Gerwick 2001). Most plasma proteins are synthesised in the liver and excreted into the peripheral blood system as part of the acute phase response (APR) (reviewed by Alexander & Ingram 1992; Bayne & Gerwick 2001; Magnadottir 2006). APR proteins such as C-reactive protein (CRP), serum amyloid P (SAP), serum amyloid A (SAA), transferrin and alpha-2-macroglobulin (α2-M) have been identified in different fish species (reviewed by Alexander & Ingram 1992; Bayne & Gerwick 2001; Magnadottir 2006). Plasma proteins may be differentially elevated or suppressed dependent upon the pathogen infecting the fish (Gerwick, Steinhauer, La Patra, Sandell, Ortuno, Hajiseyedjavadi & Bayne 2002). SAA and SAP gene expression in the gill, liver and anterior kidney of Atlantic salmon is not altered by exposure to *Neoparamoeba* spp., indicating that AGD does not stimulate a hepatic acute phase response (Bridle *et al.* 2006a).

Apart from their role in the APR, plasma proteins can also have significant biological affects on invading parasites or parasitic secretions. An important virulence factor of the parasite *Cryptobia* (*Trypanoplasma*) *salmositica* is the metalloprotease which the parasite secretes. Fish can have sub-clinical infections with the parasite but do not suffer from disease due to the neutralisation of the metalloprotease by the serum protein α2-M (Zuo & Woo 1997).

Findlay *et al* (1995) suggested that stimulation of the innate immune response may be responsible for conferring some-degree of resistance in Atlantic salmon to infection with *Neoparamoeba* spp.. The putative resistance of a group of previously exposed Atlantic salmon to subsequent infection with *Neoparamoeba* spp. was surmised to be due to stimulation of the innate immune response during the convalescence period (Findlay *et al.* 1995; Findlay & Munday 1998). However, no further work was performed to identify the pathways or components of the innate immune response that may have been responsible for the resistance. Also, Atlantic salmon injected with cytosine-phosphodiester-guanine oligodeoxynucleotides (CpGs) had significantly greater survival than untreated fish and this was purportedly due to stimulation of the innate immune responses (Bridle *et al.* 2003). Whilst these studies have suggested resistance is due to stimulation of the innate immune response, no *in vitro* or *ex vivo* studies have been performed which investigate innate immune system of Atlantic salmon to infection with *Neoparamoeba* spp..

Thesis objectives

Host responses to parasitic infections are complex and involve both cellular and humoral components of the immune system. Based upon the discussed precedencies for the role of cell-mediated and humoral immunity in the control of parasitic diseases and the evidence of host-*Neoparamoeba* spp. interactions in fish it is reasonable and prudent to pursue future research into the interactions of host immune components and *Neoparamoeba* spp.. Therefore the broad aim of this thesis was to gather information regarding the manner in which the host (Atlantic salmon) immune system components and the parasite (*Neoparamoeba* spp.) interact *in vitro*, *in vivo* and *ex vivo*.

Specific objectives of the thesis

- In chapter 2 the ability of gill cells from AGD-affected and unaffected fish to phagocytose particles, produce ROS and migrate towards a chemotactic stimulant was assessed. This study was undertaken to identify whether gill cells are capable of cellular immune responses that may protect the host against the parasite.
- The distribution and relative proportion of leucocytes in the gill of AGD-affected and unaffected fish was investigated using immunohistochemistry techniques in chapter 3. This study was undertaken to identify if any gill cell populations are affected by *Neoparamoeba* spp. attachment.
- As gill cells did not produce measurable ROS the effect of direct contact between *Neoparamoeba* spp. and anterior kidney phagocytes on ROS production was measured in chapter 4.

- Chapter 5 describes the results of study performed to investigate if *Neoparamoeba* spp. infection could alter the *in vivo* phagocytic functions of anterior kidneys cells.

- It is reported that resolution of AGD would result in resistance to re-infection and this was most likely afforded by the innate immune system (Findlay et al. 1995; Findlay & Munday 1998). Therefore the ability of anterior kidney cells isolated from fish that were responding to a second-time infection and fish that were infected for the first time to perform basic phagocytic functions was assessed and reported in chapter 6.

- In chapter 7 the results of a study which investigated the effect of plasma and mucus samples taken from AGD-affected and unaffected Atlantic salmon on *Neoparamoeba* spp. viability is reported.

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Chapter 2 Phagocytic behaviour of gill cells isolated from *Neoparamoeba* spp. infected Atlantic salmon (*Salmo salar* L.)

Manuscript in preparation

Abstract

The phagocytic activity of immune cells is high in some teleost inflammatory models. Here the ability of cells isolated from the gill of Atlantic salmon (*Salmo salar*) infected with the gill parasite *Neoparamoeba* spp., to chemotactically migrate, actively phagocytose and produce reactive oxygen species was investigated. Gill cells (GCs) and anterior kidney cells (AKCs) were isolated from healthy and amoebic gill disease (AGD)-affected Atlantic salmon and respiratory burst, phagocytosis and chemotaxis assays were performed. There was no significant intra or extracellular respiratory burst response produced by GCs, nor was there significant directional chemotactic movement of GCs. GCs engulfed yeast cells however the rate of phagocytosis was not influenced by exposure to *Neoparamoeba* spp. or the experimental time ($P > 0.05$). Infection with *Neoparamoeba* spp. did not significantly change the response of AKCs respiratory burst response, phagocytic capability or chemotactic movement ($P > 0.05$). GCs would certainly play a role in the immune response of AGD-affected fish due to their locality to the pathogen and the resulting host responses. However, data presented here suggest that whilst the isolation of viable GCs with phagocytic capacity is achievable, it remains unclear if GCs are capable of producing super oxide anion or chemotactic movement in response to *Neoparamoeba* spp. infection.

Introduction

Activation of innate cellular immune function may occur as a result of an inflammatory stimulus caused bacterial, viral or parasitic infection. In fish and mammals this stimulus often results in activation of innate cellular functions by leucocytes such as chemotaxis, respiratory burst and phagocytosis at the site of inflammation (reviewed by Suzuki & Iida 1992; Janeway & Medzhitov 2002).

Chemotaxis of leucocytes is stimulated by chemokines which may be produced by numerous cell types. Chemokines isolated from fish share a great deal of functional homology with mammalian chemokines (Secombes, Wang, Hong, Peddie, Crampe, Laing, Cunningham & Zou 2001; Lally, Al-Anouti, Bols & Dixon 2003; Peatman, Bao, Baoprasertkul & Liu 2005). The migration of leucocytes to the site of the infection often has a crucial role in resolving the infection. For example, attachment of the monogenean parasite *Neoheterobothrium hirame* to Japanese flounder (*Paralichthys olivaceus*) results in an increase in the number of leucocytes in the peripheral blood and around the site of parasite attachment. The leucocyte presence is important as they attach to the integument of the parasite, and this leads to its expulsion (Nakayasu, Tsutsumi, Oseko & Hasegawa 2005).

Phagocytosis by fish leucocytes is an integral immune response against invading pathogens and can be involved in mitigating infection. During phagocytosis, pathogens and host debris may be engulfed, stimulating the respiratory burst response and the production of reactive oxygen species (ROS), which is one of the most important functions of fish innate inflammatory cells (neutrophils and

macrophages) (Ellis 2001) and may be enhanced or suppressed in parasitised fish. European sea bass (*Dicentrarchus labrax*) parasitised by *Sphaerospora dicentrarchi* produce more ROS than non-parasitised fish (Munoz, Alvarez-Pellitero & Sitja-Bobadilla 2000). Conversely, turbot (*Scophthalmus maximus*) parasitised with the Microsporidia *Tetramicra brevifilum* produce less ROS than healthy turbot (Leiro, Iglesias, Parama, Sanmartin & Ubeira 2001).

Often these characteristic protective phagocytic behaviours are up-regulated in the tissue where the stimulus occurs and leucocyte activity in areas remote to this is either unaffected or suppressed. For example, in some fish species, such as eel (*Anguilla japonica*), carp (*Cyprinus carpio*), sea bream (*Pagellus bogaraveo*) and tilapia (*Oreochromis niloticus*), leucocytes at the site of an inflammatory stimulus are more active than leucocytes isolated from areas not associated with the stimulus (Park & Wakabayashi 1992; Itou, Iida & Kawatsu 1996; Matsuyama & Iida 1999; Matsuyama, Iida & Kurokura 1999; Kodama, Tijiwa, Moritomo & Nakanishi 2002; Serada, Moritomo, Teshirogi, Itou, Shibashi, Inoue & Nakanishi 2005).

The gill plays an important role in vaccine uptake (Moore, Ototake & Nakanishi 1998) and immunoglobulin production (Lumsden, Ostland, Byrne & Ferguson 1993; Saha, Suetake & Suzuki 2004). Plasma cells have been identified in the gills of vaccinated fish (Grontvedt & Espelid 2004) and antigen processing capabilities of gill cells have been demonstrated (Torroba, Anderson, Dixon, Casares, Varas, Alonso, Delmoral & Zapata 1993). These functions suggest that this organ plays a significant immunological role in protecting fish from disease

and that gill leucocytes are actively involved in the acquired immune response. However, whether gill leucocytes have the ability to react with innate cellular responses is unknown.

Amoebic gill disease (AGD) is associated with attachment of the protozoan parasite *Neoparamoeba* spp. to the gill of fishes (reviewed by Munday, Zilberg & Findlay 2001; Dyková, Nowak, Crosbie, Fiala, Pecková, Adams, Macháèková & Dvoráková 2005). AGD-affected fish have raised, white, mucoid patches on their gills and upon histological examination, affected gills have epithelial cell hyperplasia causing secondary lamellar fusion (Munday, Foster, Roubal & Lester 1990; Munday, Lange, Foster, Lester & Handlinger 1993; Adams & Nowak 2001; Munday *et al.* 2001; Adams & Nowak 2003; Adams, Ellard & Nowak 2004; Adams & Nowak 2004a; Adams & Nowak 2004b). An increase in the number of leucocytes in the gills of AGD-affected fish occurs as the disease progresses in severity (Munday *et al.* 1990; Munday *et al.* 1993; Adams & Nowak 2001; Munday *et al.* 2001; Adams & Nowak 2003; Adams *et al.* 2004; Adams & Nowak 2004a; Adams & Nowak 2004b). The role of these leucocytes in AGD has not been elucidated.

The respiratory burst response, chemotactic migration and phagocytic responses of fish leucocytes at the site of infection may be singularly or synergistically integral to the host immune response against pathogens, and therefore their activity is an indicator of the fish innate immune response to infection. The aim of the current study was to investigate whether cells isolated from the gills of

AGD-affected Atlantic salmon had a greater capacity for super anion production, phagocytosis and chemotaxis than GCs isolated from AGD-unaffected fish.

Materials and Methods

Fish and induction of AGD

Atlantic salmon (89.5 ± 7.2 g, n = 45) smolts obtained from a commercial salmon hatchery at Wayatinah, Tasmania (Saltas, Salmon Enterprises Tasmania) were maintained in fresh water at the University of Tasmania, Launceston. Fish were acclimated to sea water (35 ‰, 1 µm filtered) over a period of 7 d in a 500 L recirculation system connected to a biofilter. Fish were fed once daily to satiation on a commercial feed (Skretting, Hobart, Australia).

On the day the experiment commenced, 5 fish were removed for sampling and the remaining 40 fish divided equally into duplicate 500 L systems. *Neoparamoeba* spp. were isolated using a plastic adherence method (Morrison, Crosbie & Nowak 2004). One system was inoculated with *Neoparamoeba* spp. (400 cells L^{-1}) whilst the second tank of fish remained unexposed to *Neoparamoeba* spp.. Fish (n = 5 per treatment) were subsequently sampled 7 and 11 days post-*Neoparamoeba* spp. exposure (DPE).

Isolation of Gill Cells (GCs)

Fish were anaesthetised with clove oil (0.1 % v v^{-1}) and bled from the caudal vein. After the fish had been exsanguinated, the caudal vein was severed and the cardiac cavity opened. Ice-cold seawater Cortland's (SWC) saline was injected into the bulbous arteriosus (approximately 10-20 mL) using a syringe and needle. Perfused gills appeared white by the end of this process.

Immediately after perfusion, the second left gill arch was removed, placed in seawater Davidson's fixative and processed using standard histological techniques. During pilot trials the perfusion process affected the number of amoebae on the gills due to saline flushing across the gill surface however, lesions were not affected by the perfusion process and AGD-affected filaments were still enumerable. Histological sections of gills were stained with haematoxylin and eosin and the proportion of AGD-affected gill filaments relative to unaffected filaments was calculated. Immediately after the removal of the second left gill arch, the rest of the gill was placed into ice-cold Hank's balanced salt solution (HBSS) (Invitrogen Australia Pty Ltd, Mulgrave, Australia) without calcium chloride and magnesium chloride. Gills were washed in ice-cold HBSS by gentle agitation to remove mucus from the gills. This process was repeated a number of times. The absence of calcium and magnesium in the HBSS facilitated the removal of mucus.

The method of gill cell (GCs) isolation was adapted from those reported by Davidson, Lin, Secombes and Ellis (1991), Lin, Davidson, Secombes and Ellis (1998) and Lin, Ellis, Davidson and Secombes (1999). Wool filtration followed by Percoll density gradient separation of GC suspensions improved cell quality, removed cellular aggregates, mucus and epithelial cells (Lin *et al.* 1999). This was not found to be the case with regards to the methods employed in this study, and in fact significantly decreased viability was found in cells that were wool filtrated (data not shown), therefore this method was not used. Gills were

incubated in SWC supplemented with 5 % fetal calf serum (FCS), penicillin/streptomycin (P/S) (500 U mL^{-1}) (Sigma-Aldrich, Castle Hill, Australia), heparin (10 U mL^{-1}) (Sigma-Aldrich), Type 1a collagenase (0.05 mg mL^{-1}) (Sigma-Aldrich), dithiothreitol (DTT) (1 mM) (Sigma-Aldrich) and deoxyribonuclease (DN) (0.1 mg mL^{-1}) (Sigma-Aldrich) for 30 min at RT (room temperature) with shaking. Empirical experimentation identified these concentrations to be optimal for yield and cell viability (data not shown). Following incubation, gills were transferred to ice-cold HBSS and washed 3 × to again remove mucus. Gill arches were separated and macerated on a sterile 80 µm steel mesh with meshing media (SWC supplemented with 2 % FCS, 500 U mL^{-1} P/S, 10 U mL^{-1} heparin and 0.1 % L-glutamine) (Imbros Pty Ltd, Australia). The cell suspension was filtered consecutively through sterile 60 µm and 40 µm meshes, prior to 2 × washes (400 *g*, 5 min, 4 °C) in SWC washing media (supplemented with 0.1 % FCS, 500 U mL^{-1} P/S, 10 U mL^{-1} heparin and 0.1 % L-glutamine). Cells were counted and trypan blue exclusion was used to assess viability. Erythrocytes were counted as a measure of peripheral blood cell contamination, providing an indicator of gill perfusion success. Cytospin preparations of the cell isolates were prepared and stained with haematoxylin and eosin (H & E) and putative determination of cell lineage attempted. However, as it is not possible to accurately identify cell lineages without antibodies directed towards specific cell markers and this has been attempted without success (chapter 3) it was not possible to use this method to identify specific cells in the GC suspension. *In situ* histological identification of cell populations was not performed as this has been done previously by other researchers (Munday et al. 1990; Munday et al. 1993; Adams & Nowak 2001; Munday et al. 2001; Adams &

Nowak 2003; Adams et al. 2004; Adams & Nowak 2004a; Adams & Nowak 2004b).

Isolation of anterior kidney cells (AKCs)

Following gill perfusion, the anterior kidney of the fish was removed and placed into ice-cold L-15 meshing media (L-15 supplemented with 2 % FCS, 500 U mL^{-1} P/S, 10 U mL^{-1} heparin and 0.1 % L-glutamine). The anterior kidney was dissociated through a 40 µm mesh and washed 2 × in L15 washing media (as per meshing media except supplemented with 0.5 % FCS). Cytospin preparations of the cell isolates were prepared and stained with H & E.

Determination of peripheral blood leucocyte contamination

Peripheral blood leucocyte contamination was calculated by counting the number of leucocytes and erythrocytes in the peripheral blood and the number of erythrocytes in the GCs suspension. Using the following formulas and the respective gill cell yields (viable cells/gram gill tissue) the percentage of leucocytes from the peripheral blood in the gill cell isolate was able to be determined;

Leucocytes in peripheral blood	= A (cells.mL^{-1})
Erythrocytes in peripheral blood	= B (cells.mL^{-1})
Erythrocytes as percentage of gill cells	= C (%)
Proportion of A to B (A/B*100)	= D (%)
Leucocytes in gill suspension from blood	= C/D (%)

Assessment of phagocyte function

Intracellular respiratory burst

Intracellular respiratory burst was measured using the nitroblue tetrazolium (NBT) method (Secombes 1990). The original method described the use of adhered phagocytes, however pilot studies showed that the majority of gill cells would not adhere to plastic (data not shown) and therefore GCs were incubated in round-bottom tissue culture plates. Cells were incubated for 1 h with 1 mg mL^{-1} of NBT or NBT + phorbol myristate acetate (PMA) (1 μg mL^{-1}), after which cells were centrifuged at 400 × *g* for 5 min. The supernatant was removed and cells lysed for analysis of converted NBT as per standard respiratory burst procedures.

Extracellular respiratory burst

Extracellular respiratory burst production was assessed in GCs and AKCs suspensions using a standard Cytochrome C technique (Secombes, Chung & Jeffries 1988).

Chemotaxis

Chemotaxis was measured using a 48-well micro-chemotaxis chamber (Neuro Probe, Maryland, USA) and a polycarbonate filter with 5 μm pores (Neuro Probe, Maryland, USA) (Falk, Goodwin & Leonard 1980). In the bottom chamber, 25 μL of 10 % FCS in PBS (to assess directional movement) or 25 μL of PBS (to assess random movement) was placed in the wells and the chamber assembled. The upper wells were filled with either 2.5×10^5 AKCs or 2.5×10^5 GCs (50 μL volume) per well (4 wells per fish, duplicate PBS and 10 % FCS treatments). The chamber was covered and incubated at 18 °C for 60 min. Filters were removed

and non-migrated cells gently washed off with PBS. The filters were fixed with 80 % methanol and stained in Quick-Dip. The number of migrated cells per field of view (FOV) in at least 10 random fields on the bottom surface of the filter was counted using a light microscope at 1000 × magnification. Migration ratio was determined as the number of cells FOV^{-1} exhibiting directional movement / number of cells FOV^{-1} exhibiting random movement.

Phagocytosis

The phagocytosis assay was performed according to published methods (Thompson, Lilley, Chen, Adams & Richards 1999; Polonio, Wolke, MacLean & Sperry 2000). Briefly, AKCs and GCs were incubated with 1×10^7 Congo red stained yeast cells (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) (100 µL). The cells were incubated for 1 h at 18 °C, fixed with 80 % methanol and stained with Quick-Dip (Histo Labs, Riverstone, Australia). Cells were examined by light microscopy using 1000 × magnification. Phagocytic rate (PR) was evaluated by estimating the mean proportion of cells containing yeast, in a random count of 100 cells performed in duplicate. The phagocytic index (PI) was determined by estimating the mean number of yeast within each cell.

Statistical Analyses

Data were analysed using a two-way analysis of variance (ANOVA) with time and exposure status as the test variables. GCs and AKCs results were not compared with each other. Data were tested for homogeneity of variance using Levene's test for homogeneity, if variances were non-homogenous then they were log transformed for analysis. Tukey's test was used to differentiate between means and differences were considered significant at the $P < 0.05$ level.

Histology data were analysed using a one-way ANOVA and Tukey's test to identify significant differences between proportions of AGD-affected filaments over exposure duration. Data are presented as mean ± standard error (SEM).

Results

Induction of AGD and histological analysis of gills

The proportion of AGD-affected gill filaments from fish in the *Neoparamoeba* spp. exposed group was 0, 9 and 43 % on 0, 7 and 11 DPE respectively (Figure 2-1). Fish in the control group that had not been exposed to *Neoparamoeba* spp. did not have any histological evidence of AGD throughout the study (Figure 2-1).

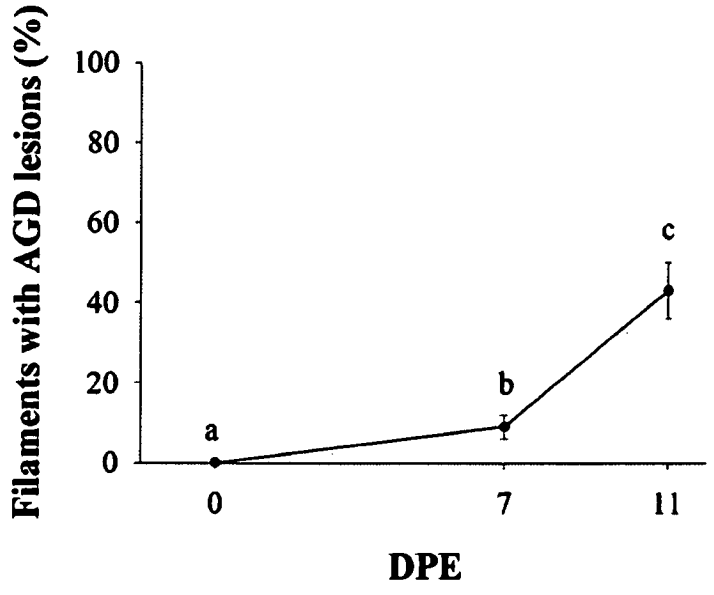


Figure 2-1 Proportion of gill filaments with AGD-associated lesions from *Neoparamoeba* spp. exposed (infected) Atlantic salmon over exposure time of 0, 7 and 11 days. DPE = days post-*Neoparamoeba* spp. exposure.. No signs of AGD or AGD-associated lesions were found on the gill of unexposed fish (data not shown). Statistical analysis performed on data from infected fish only. Different letters indicate significant differences ($P < 0.05$) over exposure time for the exposed (infected) group, n = 5 fish per sampling time point.

There was evidence of edema in the perfused gills. There were no amoebae on the gills of AGD-affected fish that had been perfused and was probably due to the perfusion process where the saline flushed across the surface of the gill causing dislodgment of the parasites. Despite the removal of amoebae, hyperplasia was still evident in perfused gills allowing for identification of AGD-affected areas of the gill (Figure 2-2). There were few leucocytes seen in the central venous sinus (CVS) which supports the use of gill perfusion to remove contaminating peripheral blood leucocytes and provides further evidence that the cells in the GCs suspension were gill tissue resident cells and not contaminants from the peripheral blood.

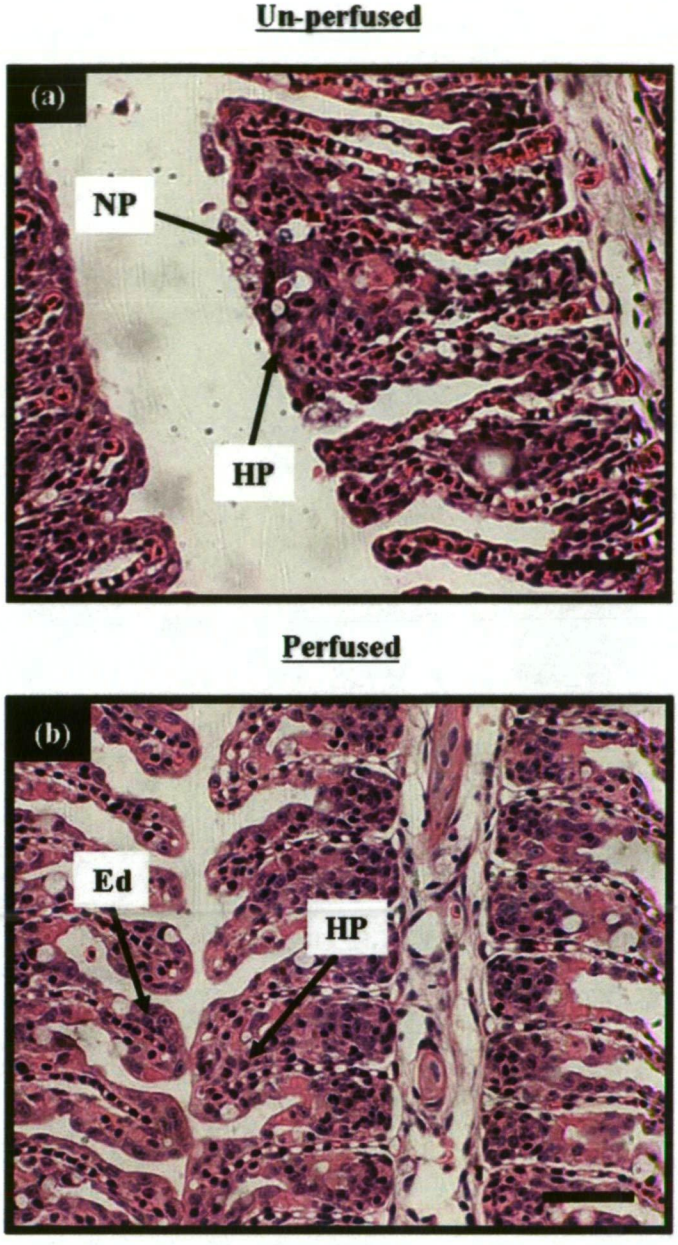


Figure 2-2 Histology from fish with amoebic gill disease. (a) Un-perfused gill histology and (b) perfused gill histology. Sections were stained with haematoxylin and eosin. NP = Amoebae trophozoite, HP = hyperplasia, Ed = edema caused by saline perfusion. Note absence of amoebae on gills of perfused fish due to saline flooding of gills during perfusion technique, it was thought that this removed the amoebae due to the osmolality of the saline being lower than sea water and/or a flushing effect. Bar = 20 µm.

Isolation of cells from gills and anterior kidney

The number of viable cells isolated per gram of gill tissue did not change during the course of the infection regardless of exposure to *Neoparamoeba* spp. or

duration of exposure time ($P > 0.05$). The mean viability of isolated GCs was 71.1 ± 3.5 % (mean ± SE, $n = 25$) and the mean total number of viable cells isolated was $1.30 \pm 0.1 \times 10^7$ per gram of gill tissue (mean ± SE, $n = 25$) (Figure 2-3). The mean viability of AKCs isolated was 94.3 ± 1.1 % (mean ± SE, $n = 25$).

Contamination of gill cell isolate with peripheral blood cells

Very few erythrocytes were found in the GCs suspensions, indicating few leucocytes from the peripheral blood were in the GCs suspension and that the perfusion process was successful (Figure 2-4 a-d). Leucocytes and other cell-lineages were visible in H & E stained cytospins of isolated AKCs and GCs (Figure 2-5).

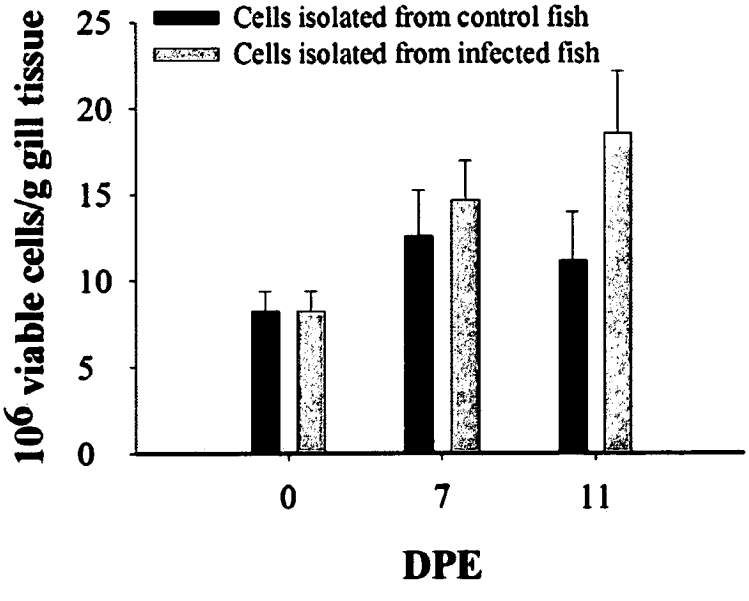


Figure 2-3 Total number of viable cells corrected for tissue weight isolated from the perfused gill of both AGD-affected (infected) and unaffected (control) Atlantic salmon. DPE = days post-*Neoparamoeba* spp. exposure.

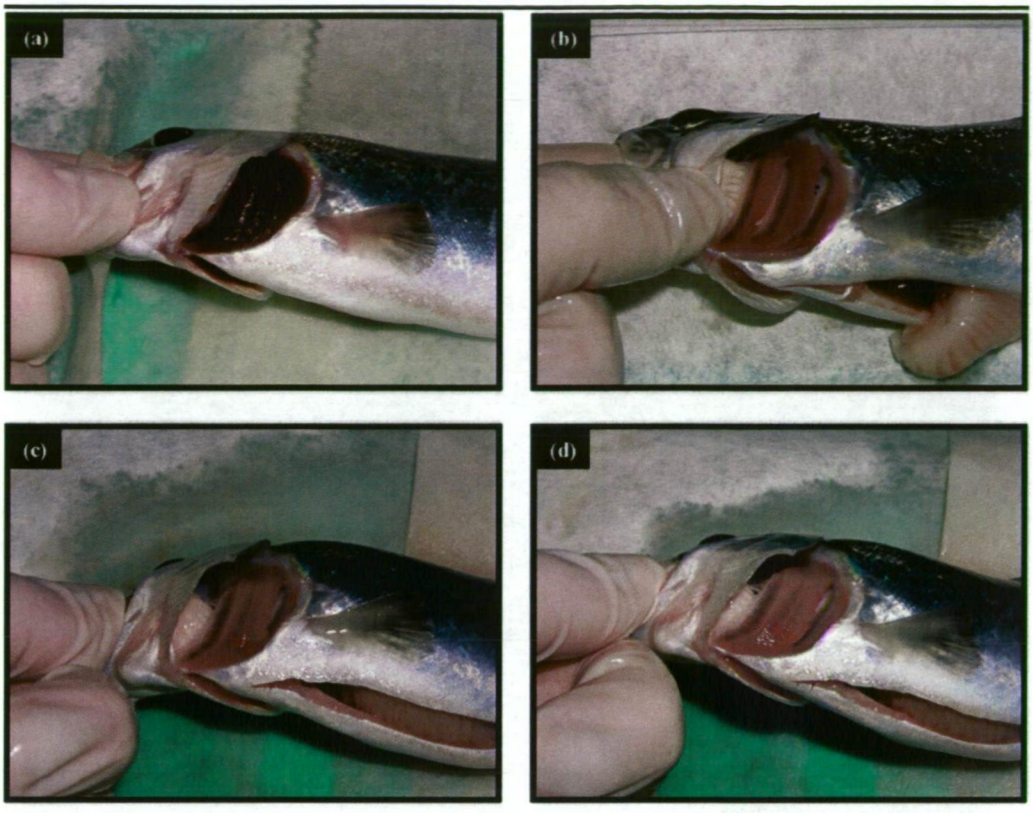
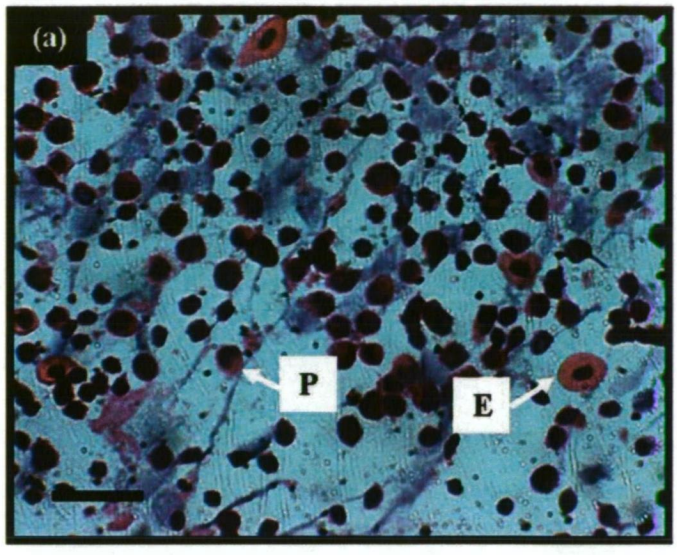


Figure 2-4 The progression of gill perfusion; gill prior to perfusion (a), during perfusion process (b), mid-way during perfusion (c), final perfused gill note white colour of gill (d).

Exposure to *Neoparamoeba* spp. and the sample time at which the cells were isolated did not affect the proportion of leucocytes or erythrocytes in the peripheral blood or GCs suspensions ($P > 0.05$) (data not shown). For this reason the means are reported based upon the average of the data. Atlantic salmon blood contained $2.51 \pm 0.1 \times 10^9$ erythrocytes per mL (mean ± SE, $n = 25$) and $2.94 \pm 0.3 \times 10^8$ leucocytes per mL (mean ± SE, $n = 25$). The ratio of leucocytes to erythrocytes was 11.7 ± 2.2:1 (mean ± SE, $n = 25$). The perfused gill cell suspensions contained 1.04 ± 0.3 % erythrocytes (mean ± SE, $n = 25$) which may have come from peripheral blood, so only 0.12 ± 0.04 % (mean ± SE, $n = 25$) of the cells in the gill suspensions could have originated from the peripheral blood, and this small proportion of contaminating cells was considered negligible.

Anterior kidney cells



Gill cells

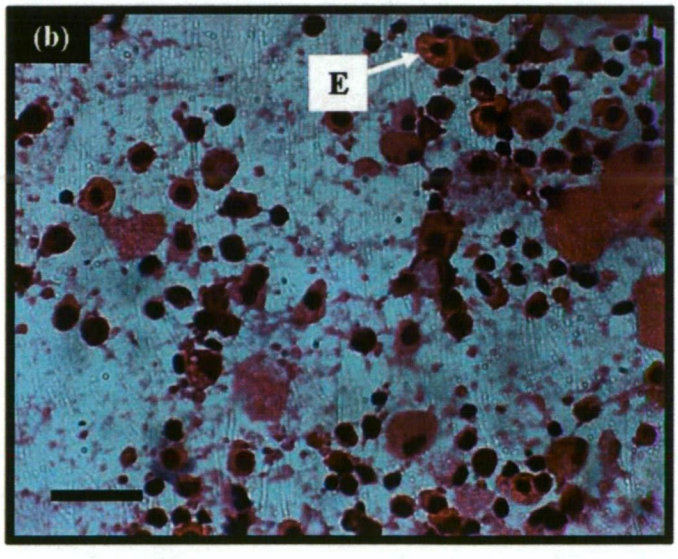


Figure 2-5 Cytospin preparations for the identification of cells in the anterior kidney (a) and gill cell (b) isolations. The cytospin preparations were stained with haematoxylin and eosin. E = erythrocyte, P = anterior kidney cell with morphological characteristics consistent with a phagocyte. Bar = 20 μm.

Assessment of phagocyte function

Respiratory burst response

AKCs and GCs isolated from each fish were assayed in parallel with the AKCs being used as assay controls. The presence of NBT granules within the cells was

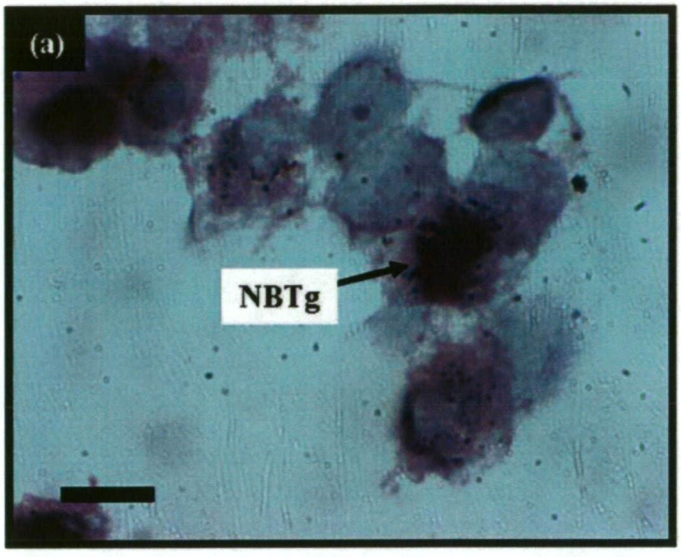
confirmed visually by making a cytospin preparation of cells prior to addition of the lysis buffer. The cytospins were stained with H & E and examined using an inverted microscope (Figure 2-6).

There was no difference in the measurable intracellular respiratory burst response of resting and PMA-stimulated GCs despite a measurable "resting" response being detected. This suggests that either the response was not a "true" respiratory burst or that the cells were not stimulated by PMA. AKCs responded to stimulation with PMA with an approximate 2-fold increase in intracellular ROS production (Figure 2-7 a,b). The increase in superoxide production by PMA-stimulated AKCs compared to unstimulated AKCs was significantly different ($P < 0.05$) 0, 7 and 11 DPE for both AGD-affected and unaffected groups. There were no differences ($P > 0.05$) in intracellular ROS production between AGD-affected and unaffected groups or over sampling time for either GCs or AKCs (Figure 2-7 a,b).

The extracellular respiratory burst responses of AKCs and GCs were similar to that of the intracellular respiratory burst responses. Gill cells produced a measurable response however they were not stimulated by PMA to produce an enhanced response above basal levels (Figure 2-8 a,b). AKCs produced a measurable resting response and an enhanced PMA-stimulated response, approximately 2-fold over resting levels. The increase in superoxide production by PMA-stimulated AKCs compared to unstimulated AKCs was significantly different ($P < 0.05$) 0, 7 and 11 DPE for both AGD-affected and unaffected groups. There were no differences ($P > 0.05$) in extracellular ROS production

between AGD-affected and unaffected groups or over sampling time for either GCs or AKCs (Figure 2-8 a,b).

Anterior kidney cells



Gill cells

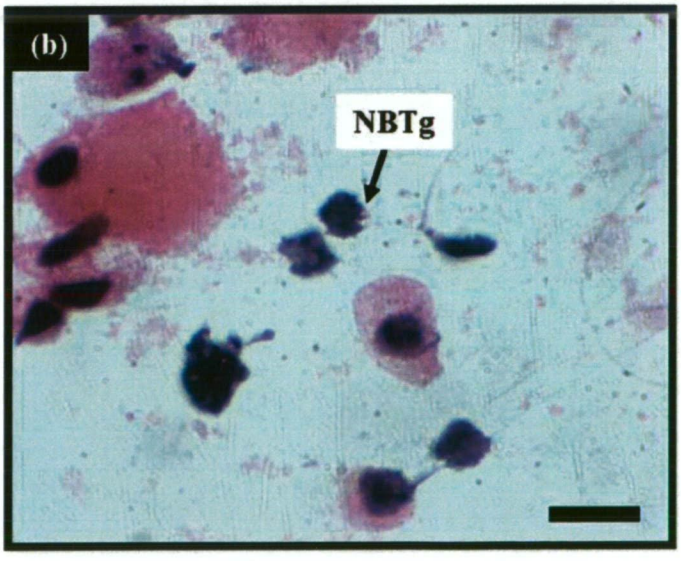


Figure 2-6 Cytospin of anterior kidney cells (a) and isolated gill cells (b) during assessment of intracellular respiratory burst activity. Cells were incubated with 100 µL of nitroblue tetrazolium salts (1 mg mL^{-1}) and PMA (1 µg mL^{-1}) for 1 h. An aliquot of the cell suspension was cytospin and stained with haematoxylin and eosin. NBTg = Nitroblue tetrazolium granules. Bar = 20 µm.

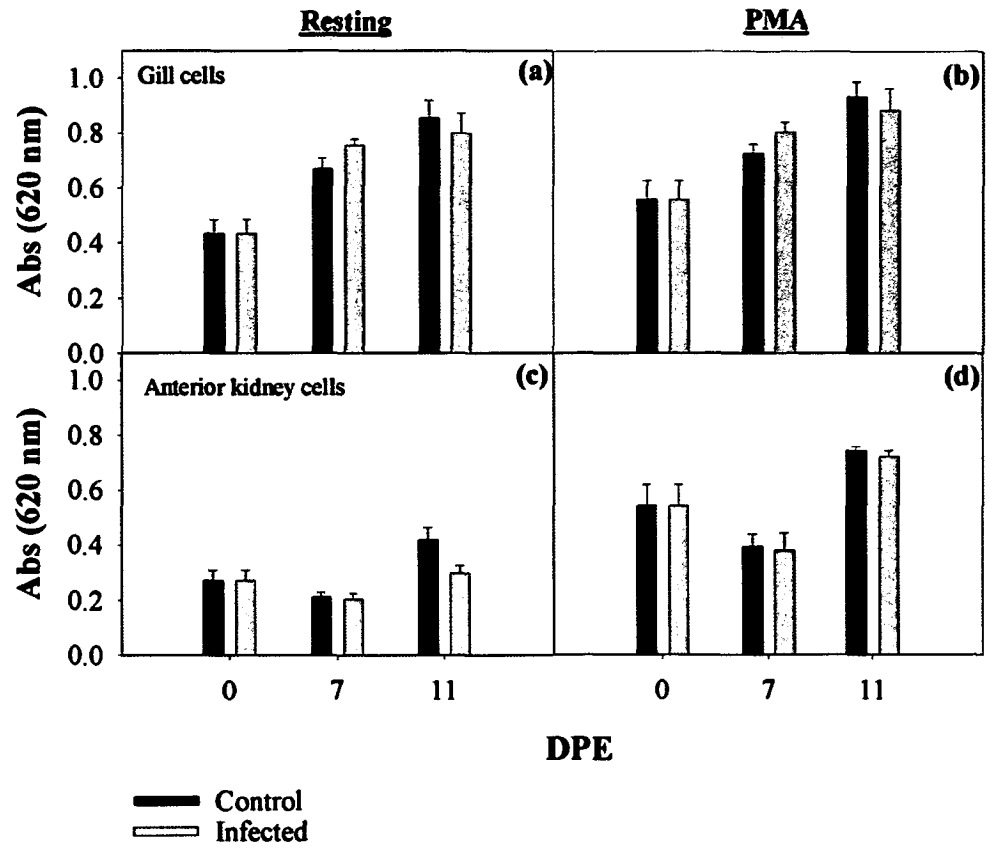


Figure 2-7 Intracellular respiratory burst of cells isolated from the anterior kidney and gill of AGD-affected (infected) and unaffected (control) Atlantic salmon. Resting (unstimulated) and PMA-stimulated respiratory burst responses were measured in cells from both anterior kidney and gill. DPE = days post-*Neoparamoeba* spp. exposure. (a) resting gill cell respiratory burst response, (b) PMA-stimulated gill cell respiratory burst response, (c) resting anterior kidney cell respiratory burst response, (d) PMA-stimulated anterior kidney cell respiratory burst response. Data are presented as means ± standard error, $n = 5$ fish per group, per sample. No significant difference ($P > 0.05$) was found over exposure time or between infected and control groups for both AKCs and GCs. AKCs resting and PMA-stimulated respiratory burst were significantly different ($P < 0.05$) 0, 7 and 11 DPE for both exposure groups (significant differences not shown). There was no significant difference between resting and PMA-stimulated ROS production for GCs ($P > 0.05$)

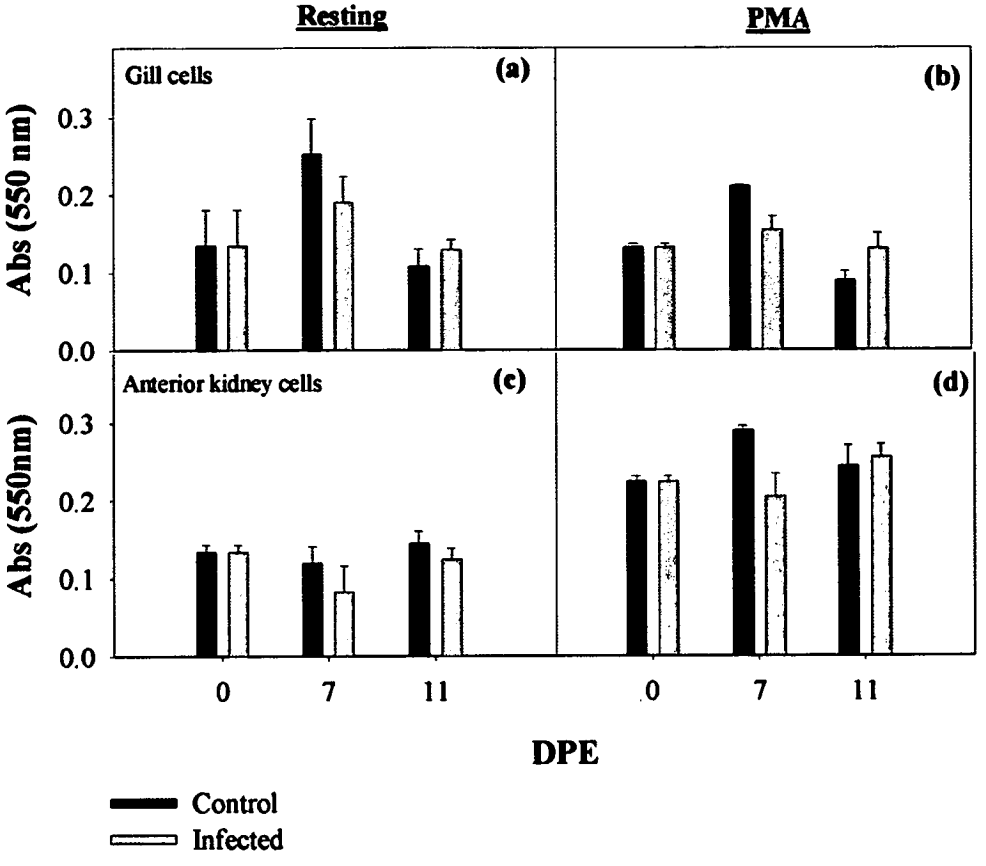


Figure 2-8 Extracellular superoxide release by cells isolated from the anterior kidney and gill of AGD-affected (infected) and unaffected (control) Atlantic salmon. Resting (unstimulated) and PMA-stimulated respiratory burst responses were measured from cells isolated from both anterior kidney and gill. DPE = days post-*Neoparamoeba* spp. exposure. (a) = resting gill cell respiratory burst response, (b) = PMA-stimulated gill cell respiratory burst response, (c) = resting anterior kidney cell respiratory burst response, (d) = PMA-stimulated anterior kidney cell respiratory burst response. Data are presented as means ± standard error, n = 5 fish per group, per sample. No significant difference ($P > 0.05$) was found over exposure time or between infected and control groups for both anterior kidney cells and gill cells. AKCs resting and PMA-stimulated respiratory burst were significantly different ($P < 0.05$) 0, 7 and 11 DPE for both exposure groups (significant differences not shown). There was no significant difference between resting and PMA-stimulated ROS production for GCs ($P > 0.05$).

Chemotaxis

Significantly more AKCs migrated towards FCS compared to the number of cells that migrated towards PBS ($P < 0.05$) (Figure 2-9). There was no significant difference in the chemotactic migration of AKCs isolated from AGD-affected and

unaffected fish ($P > 0.05$). Nor was there a significant difference in the chemotactic migration of AKCs isolated from AGD-affected or unaffected fish during the experiment (Figure 2-9). GCs did not exhibit significant directional migration in response to 10 % FCS. There were no differences ($P > 0.05$) in the chemotactic movement of GCs isolated from AGD-affected and unaffected fish. The duration of the experiment also did not ($P > 0.05$) affect the chemotactic ability of GCs isolated from AGD-affected and unaffected fish (Figure 2-9). Upon visual examination of the chemotactic filters, an occasional cell was identified as having migrated through the membrane, however this was very rare (Figure 2-10).

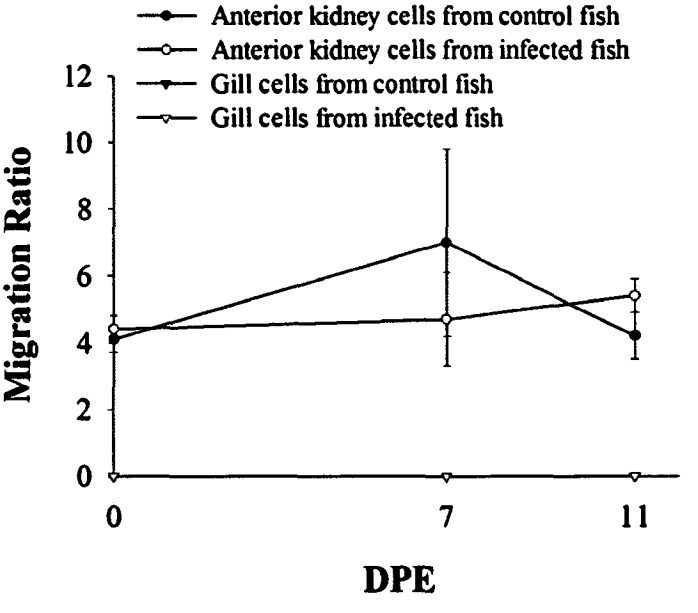
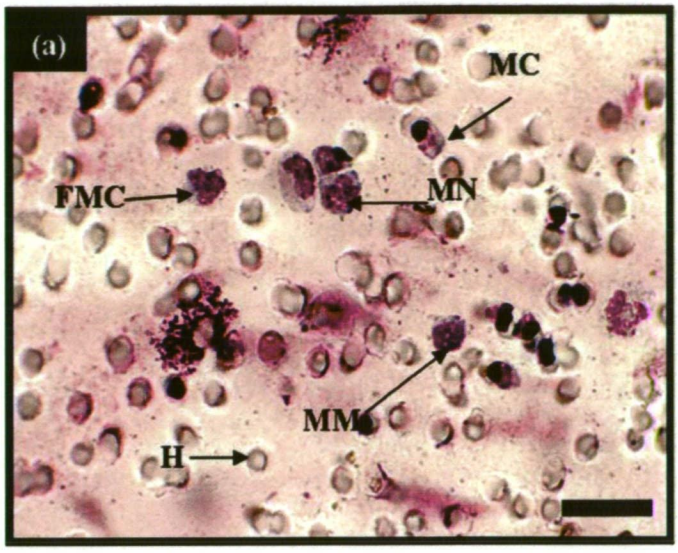


Figure 2-9 Migration ratio of AKCs and GCs isolated from AGD-affected (infected) and unaffected (control) Atlantic salmon. Migration ratio is the number of cells migrating through a filter with 5µm pore size towards 10 % fetal calf serum / number of cells migrating through a filter with 5µm pore size towards phosphate buffered saline. DPE = days post-Neoparamoeba spp. exposure. Data are presented as means ± standard error, n = 5 fish per group, per sample. No significant difference ($P > 0.05$) was found over exposure time or between infected and control groups for both AKCs and GCs. Migration ratio results of GCs isolated from control fish are hidden behind results of GCs from infected fish due to the values overlapping.

Phagocytosis

The phagocytic responses (rate and index) of AKCs isolated from AGD-affected and unaffected Atlantic salmon were not significantly different ($P > 0.05$) (Figure 2-11 and Figure 2-12 respectively). The phagocytic rate and index of GC isolates remained constant between exposure groups and over exposure time. The phagocytic rate and index of GCs isolated from AGD-affected and unaffected Atlantic salmon were not altered by duration of exposure or exposure to *Neoparamoeba* spp. ($P > 0.05$) (Figure 2-11 and Figure 2-12 respectively). There was a large degree of variation between individual fish with regard to the proportion of GCs that were actively phagocytosing yeast cells and therefore large standard errors of mean. This variation may have been a result of the number or cell lineage of GCs involved in phagocytosis and/or the quality and variation in viability of the GCs (Figure 2-13).

Anterior kidney cells



Gill cells

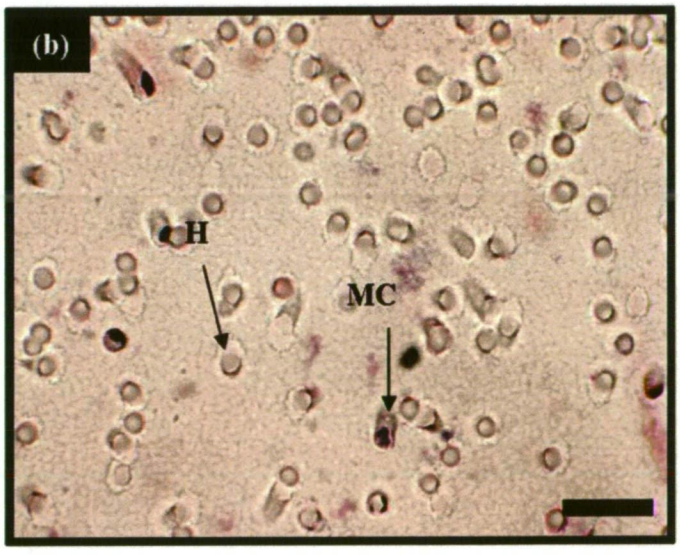


Figure 2-10 Chemotaxis of anterior kidney (a) and gill (b) cells through 5 μm filters towards 10 % FCS. MC = migrating cell moving through pore of filter, FMC = fully migrated cell, MN = migrated neutrophil, MM = migrated macrophage, H = membrane pore hole. Note lack of fully migrated GCs on filter. Bar = 10 μm.

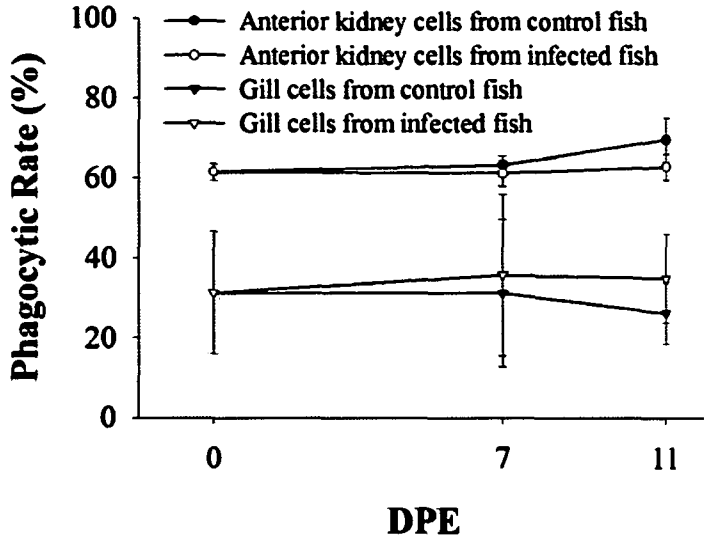


Figure 2-11 Phagocytic rate (number of cells actively phagocytosing Congo stained yeast / total number of cells × 100) of cells isolated from the anterior kidney and gill of AGD-affected and unaffected Atlantic salmon. Data are presented as means ± standard error, n = 5 fish per group, per sample. No significant difference ($P > 0.05$) was found over exposure time or between infected and control groups for both AKCs and GCs. DPE = days post-*Neoparamoeba* spp. exposure.

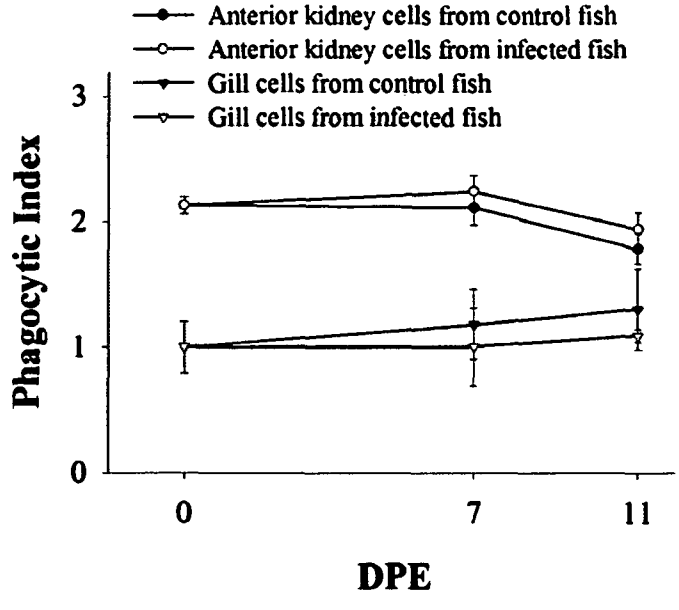
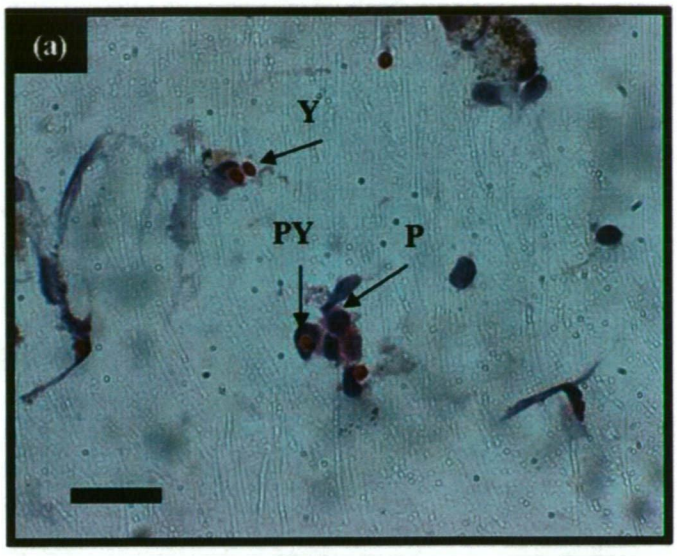


Figure 2-12 Phagocytic index (number of yeast cells phagocytosed / actively phagocytosing cell) of anterior kidney and gill cells isolated from AGD-affected and unaffected Atlantic salmon. Data are presented as means ± standard error, n = 5 fish per group, per sample. No significant differences ($P > 0.05$) were found over exposure time or between infected and control groups for both AKCs and GCs. DPE = days post-*Neoparamoeba* spp. exposure.

Anterior kidney cells



Gill cells

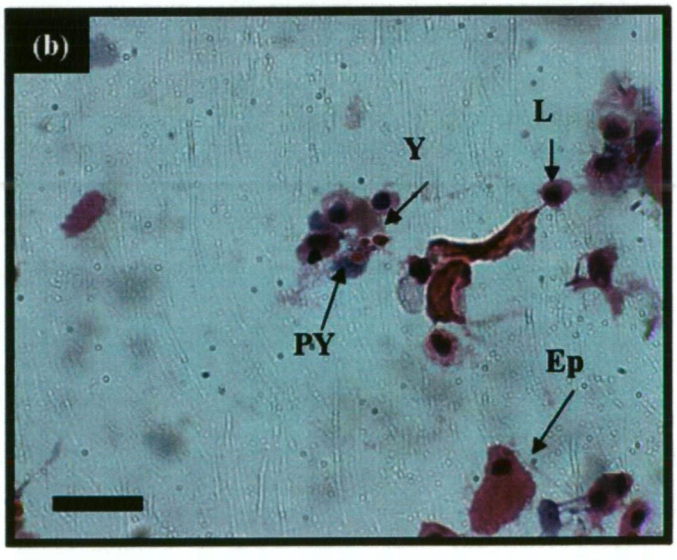


Figure 2-13 Haematoxylin and eosin stained cytospin of anterior kidney cells (a) and gill cells (b) during phagocytosis of Congo stained yeast cells. P = phagocyte, Y = yeast cell, PY = anterior kidney or gill cell phagocytosing a yeast. Bar = 20μm.

Discussion

During parasitic diseases in fish the quantity of leucocytes in the affected-areas will often increase during infection and the cellular activities at these sites may also be enhanced. An infiltration of leucocytes into the gills of *Neoparamoeba* spp. infected fish was noted during AGD (Adams & Nowak 2003; Adams *et al.* 2004; Adams & Nowak 2004a; Adams & Nowak 2004b). It was hypothesised that the ability of GCs from AGD-affected fish to produce a respiratory burst response, phagocytose particles and chemotactically migrate may be important for the development of protective immunity as cells at the site of infection are often more capable of performing these functions than cells from uninflamed sites. When the respiratory burst response of leucocytes isolated from the inflamed peritoneal cavity of carp was assessed it was much greater than the response of anterior kidney cells from the same fish or cells isolated from uninflamed peritoneal cavities (Serada *et al.* 2005). Also, increases in alveolar macrophage numbers and increased oxidative burst of cells isolated from the lung of guinea-pigs affected by pulmonary stage *Trichinella spiralis* infection compared to healthy guinea pigs have been reported (Dzik, Golos, Jagielska, Kapala & Walajtys-Rode 2002; Dzik, Zielinski, Golos & Walajtys-Rode 2006). Those results indicated that cells at the site of the inflammation were more active than cells in uninflamed areas. However, in the present study GCs isolated from both groups of fish responded similarly with respect to ROS production, phagocytic ability and chemotaxis.

The infiltration of leucocytes into the gills of *Neoparamoeba* spp. infected fish (Adams & Nowak 2003; Adams *et al.* 2004; Adams & Nowak 2004a; Adams &

Nowak 2004b) presumably indicates their migration from the host haematopoietic tissue, blood or local immune cell populations. Migration of leucocytes, especially neutrophils to the site of an inflammatory stimulus usually results in a population of cells with high migratory activity and a high proportion of neutrophils or macrophages (Matsuyama & Iida 1999; Matsuyama *et al.* 1999). However, this response is short-term and occurs soon after exposure to the irritant. Neutrophils isolated from the inflamed swim bladder of carp and red seabream (*Pagrus major*) had the highest chemotactic activity 48 hours after the swim bladder was injected with the irritant (formalin-killed *Escherichia coli*). However, the migratory response had declined by 60 and 70 hours post-injection (Matsuyama *et al.* 1999). In the present study GCs from both healthy and AGD-affected fish did not exhibit chemotactic migration. It has been reported that cells isolated from the gill of rainbow trout (*Oncorhynchus mykiss*) were chemotactically stimulated by 2 % rainbow trout serum but not with lower concentrations of serum (Lin *et al.* 1999). The discrepancies between the results of Lin *et al* (1999) and those of the current study may be due the different chemotactic stimuli used, however in the current study AKCs chemotactically migrated towards the FCS which would indicate that FCS is an attractant for migration of cells isolated from Atlantic salmon. Whether the lack of chemotaxis by GCs was because the peak chemotactic activity had passed, the GCs were not predominately leucocytic or they were not chemotactically adept is unknown.

Intra and extracellular production of ROS by GCs was detectable however the GCs were unable to be stimulated with PMA to produce ROS. AKCs isolates on the other hand doubled the amount of ROS produced upon PMA stimulation.

Visual examination of smears of GCs used in the intracellular respiratory burst assay did not show NBT conversion in phagocyte-like cells and the relative proportion of phagocytes in the gill cell suspension appeared low. It has been identified that the major leucocyte population in rainbow trout gill cell isolates are lymphocytes (Lin *et al.* 1999) which do not produce ROS. Therefore based upon the reported leucocyte proportions in GCs isolates (Lin *et al.* 1999) and the observations of the GCs suspensions in this study, ROS production and yeast cell phagocytosis in the present study may have been due to non-leucocytic cells present in the suspension.

Generation of superoxide anion by mammalian cells other than leucocytes has been reported, and include endothelial (Hohler, Holzapfel & Kummer 2000), lens epithelial, (Rao, Maddala, John & Zigler 2004) mesangial (Kwan, Wang, Munk, Xia, Goldberg & Whiteside 2005), vascular smooth muscle (Janiszewski, Lopes, Carmo, Pedro, Brandes, Santos & Laurindo 2005), fibroblasts (Lijnen, Papparella, Petrov, Semplicini & Fagard 2006), thyroid (De Deken, Wang, Many, Costagliola, Libert, Vassart, Dumont & Miot 2000) and spermatozoa (Vernet, Fulton, Wallace & Aitken 2001; Sabeur & Ball 2006). There have been no reports in the literature with regards to the production of ROS by cell types other than leucocytes isolated from fish, however, there have also been no reported studies investigating this. In the present study cells in the GCs suspension other than leucocytes may have been responsible for producing ROS and therefore converting NBT and cytochrome C. Other cell lineages such as endothelial, fibroblasts, epithelial, mucus and chloride cells may have contributed or in fact been the predominant ROS producers.

Leucocytes produce ROS only in response to antigenic stimulation as it is a protective measure (Babior 1999). However, production of ROS by other cell types may occur during normal physiological functions such as transcriptional activation, cell proliferation and apoptosis (reviewed by Forman & Torres 2002). In the current study ROS production by non-leucocytic GCs may have occurred as a physiological response to gill epithelial cell hyperplasia or as a product of normal fish homeostasis. For example murine spermatozoa have an on-going requirement for ROS and its production occurs perpetually at background levels (Vernet *et al.* 2001). The inability of the GCs in the current study to be stimulated by PMA to produce increased levels of ROS may be due to a lack of protein kinase C (PKC) receptors (Nauseef, Volpp, McCormick, Leidal & Clark 1991; Tudan, Jackson, Pelech, Attardo & Burt 1999) or the manner in which the NADPH-oxidase complex is activated in non-phagocytic cells (Vernet *et al.* 2001; De Coursey & Ligeti 2005; Sabeur & Ball 2006). For example rat spermatozoa do not require that the NADPH-oxidase complex to be activated for ROS production (Vernet *et al.* 2001).

A proportion of GCs were able to engulf yeast cells, indicating that there were viable, functioning phagocytic cells within the GCs suspensions (Figure 2-13). However as the GCs were incapable of PMA-stimulated ROS production and chemotaxis, it may be hypothesised that the phagocytic GCs were not all professional phagocytes (macrophages/monocytes and neutrophils) but other cell lineages for example epithelial and/or endothelial. Cell types other than the professional phagocytes have been identified as being capable of antigen uptake

in fish. Scavenger endothelial cells have been identified in the kidney (both anterior and posterior) (Smedsrod, Gjoen, Sveinbjornsson & Berg 1993; Dannevig, Lauve, Press & Landsverk 1994; Seternes, Sorensen & Smedsrod 2002), heart (Sorensen, Melkko & Smedsrod 1998), spleen (Dalmo, Ingebrigtsen & Bogwald 1997), gill and liver of fishes (Seternes *et al.* 2002). Whilst most of these scavenger endothelial cells (Dannevig *et al.* 1994; Sorensen *et al.* 1998; Seternes *et al.* 2002) are involved in macromolecule uptake there is also evidence for the involvement of endothelial (Dannevig *et al.* 1994) and epithelial cells in particulate antigen up-take (Torroba *et al.* 1993; Moore *et al.* 1998; Glenney & Petrie-Hanson 2006). Microspheres have been identified in epithelial cells from the gill and skin of rainbow trout (Moore *et al.* 1998) and the gill, torso, fin and nares of Channel catfish (*Ictalurus punctatus*) (Glenney & Petrie-Hanson 2006) following immersion of the fishes in the microspheres. Pillar cells have also been identified as a specific cell type within the gill that is capable of phagocytosis (Chilmonczyk & Monge 1980). Together with the observations from this current study it is hypothesised that the GCs engulfing the yeast cells were not all professional phagocytes but possibly epithelial cells. This is supported by their tissue of origin (the gill) being composed predominately of epithelial cells and the fact that AGD-affected fish in this study did not respond to infection with an intense localised leucocytic reaction as is seen in other parasitic diseases of fish (Dezfuli, Giari, Konecny, Jaeger & Manera 2003; Yasutake & Elliott 2003) and mammals (Dzik *et al.* 2002; Dzik *et al.* 2006).

In summary viable, functioning GCs were isolated from both AGD-affected and unaffected fish and they responded similarly with respect to ROS production,

chemotaxis and phagocytosis. This suggests that under the conditions described, *Neoparamoeba* spp. infection did not alter the ability of GCs to perform the mentioned functions compared to GCs from healthy fish. It is not known why GCs did not respond to PMA stimulation with increased ROS production or why they were unable to chemotactically migrate, but it may have been due to stimulation of the cells during isolation, mucus products in the chemotactic assay cell samples, differing requirements of GCs compared to AKCs or a low proportion of phagocytes in the GCs suspension. Certainly compared to the responses of fish to other parasitic infections (Dezfuli *et al.* 2003) the leucocytosis is not extensive and potentially this may be the defining variable in assessing the ability of gill phagocytes to produce measurable superoxide anions, migrate or phagocytose as their specific numbers are below assessable limits.

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Chapter 3 Identification and distribution of leucocytes in the gills of AGD-affected Atlantic salmon (*Salmo salar* L.)

Manuscript in preparation

Abstract

It has been reported that Amoebic gill disease (AGD)-affected Atlantic salmon (*Salmo salar*) have infiltrations of leucocytes in the affected gills and develop interlamellar vesicles in AGD-affected areas. Atlantic salmon were experimentally infected with *Neoparamoeba* spp. (100 cells L^{-1}) to induce AGD. Fish were sampled 0, 8, 16, 24 and 32 days post-exposure (DPE) with gills taken for immunohistochemical studies. Three antibodies that bind to immunoglobulin (Ig), lysozyme and iNOS (inducible nitric oxide synthase) were characterised and optimised for use during this study. We report the presence of immunoglobulin bearing cells in the gills of AGD-affected and unaffected Atlantic salmon and also cross-reactivity of antibodies that bind mammalian antigens with Atlantic salmon leucocytes. Rabbit anti-Atlantic salmon Ig antibody that bound the heavy chain region of purified Atlantic salmon Ig was used to identify Ig bearing cells in the gills of Atlantic salmon during infection with *Neoparamoeba* spp.. There was no significant difference ($P > 0.05$) in the number or distribution of Ig bearing cells in the gills of uninfected and infected Atlantic salmon. The majority of Ig bearing cells were found in the tips of the filaments. In addition, the presence of Ig bearing cells in interlamellar vesicles was studied using archived gill material, however no Ig bearing cells were identified in these vesicles. A commercial rabbit anti-lysozyme antibody bound a number of proteins in Atlantic salmon serum but no protein with a molecular weight consistent with lysozyme. A commercial rabbit anti-rat iNOS antibody reported to bind rainbow trout (*Oncorhynchus mykiss*) iNOS did not bind an Atlantic salmon peptide consistent with the predicted molecular weight of iNOS. These data identify the gill as a site of Ig bearing cells. However, given the indifferent response of these cells

throughout *Neoparamoeba* spp. infection their role in AGD pathogenesis is unknown.

Introduction

An inflammatory reaction to infection in fish usually begins with an acute phase response which includes an infiltration of leucocytes to the site of infection (reviewed by Suzuki & Iida 1992; Bayne & Gerwick 2001). Once at the site of infection leucocytes may produce reactive nitrogen intermediates (RNI) (Dezfuli, Giari, Konecny, Jaeger & Manera 2003) and reactive oxygen species (ROS) (Park & Wakabayashi 1992; Itou, Iida & Kawatsu 1996; Matsuyama & Iida 1999; Matsuyama, Iida & Kurokura 1999; Kodama, Tijiwa, Moritomo & Nakanishi 2002; Serada, Moritomo, Teshirogi, Itou, Shibashi, Inoue & Nakanishi 2005), important protective molecules. Fish may respond to parasitic infections by increasing the number of eosinophilic granular cells (EGC) at the site of infection, often these cells become a dominant lineage around the parasite (Dezfuli, Simoni, Rossi & Manera 2000). The acquired immune response is initiated secondary to the innate immune response and an influx of immunoglobulin (Ig) bearing cells such as B lymphocytes to the site of the infection may occur (Bermudez, Vigliano, Marcaccini, Sitja- Bobadilla, Quiroga & Nieto 2006). Infection of the gills of fishes with *Neoparamoeba* spp. results in a response that is characterised macroscopically by the presence of raised, white, mucoid patches on the gills and is termed amoebic gill disease (AGD) (reviewed by Munday, Zilberg & Findlay 2001). AGD lesions are composed of hyperplastic epithelium which leads to lamellar fusion and an infiltration of leucocytes into the central venous sinus (Munday, Foster, Roubal & Lester 1990; Munday, Lange, Foster, Lester & Handlinger 1993; Adams & Nowak 2001; Munday *et al.* 2001; Adams & Nowak 2003; Adams, Ellard & Nowak 2004; Adams & Nowak 2004a; Adams & Nowak 2004b). Interlamellar vesicles containing leucocytes have been identified in

association with AGD lesions (Roubal, Lester & Foster 1989; Adams & Nowak 2001; Adams & Nowak 2003; Bridle, Butler & Nowak 2003; Adams & Nowak 2004a; Adams & Nowak 2004b) however, the only cells specifically characterised in gill lesions are those bearing major histocompatibility complex class II β-chain (MHC-II) (Morrison, Koppang, Hordvik & Nowak 2005).

AGD associated lesions may be an area of significant immunological importance with regard to antigen uptake, processing and presentation and consequentially the development of a systemic immune response (Morrison *et al.* 2005). Atlantic salmon (*Salmo salar*) are capable of producing anti-*Neoparamoeba* spp. antibodies during infection with *Neoparamoeba* spp. (Findlay, Helders, Munday & Gurney 1995; Akhlaghi, Munday, Rough & Whittington 1996; Gross, Carson & Nowak 2004a). Thus, the presence of Ig bearing cells in the gills of AGD-affected fish and their location relative to lesions and associated *Neoparamoeba* sp. was investigated.

Nitric oxide (NO) production by immune cells plays an important role in the immune response of fish to invading pathogens by its toxic effects on pathogens (Campos-Perez, Ellis & Secombes 2000; Acosat, Real, de Galarreta, Diaz, Padilla & Ellis 2003). The up-regulation of inducible nitric oxide synthase (iNOS) as a result of parasitic infection in fish usually occurs locally at the site of the infection and not in organs such as the anterior kidney, liver or spleen (Magarinos, Romalde, Santos, Casal, Barja & Toranzo 1994; Campos-Perez, Ward, Grabowski, Ellis & Secombes 2000; Sigh, Lindenstrom & Buchmann 2004a;

Sigh, Lindenstrom & Buchmann 2004b; Bridle, Morrison & Nowak 2006b), possibly so that NO can have a direct contact with the pathogen. For example during cutaneous infection of rainbow trout (*Oncorhynchus mykiss*) with the parasitic ciliate *Ichthyophthirius multifiliis*, iNOS is up-regulated only in the skin and not in the anterior kidney or spleen (Sigh *et al.* 2004b). Furthermore, iNOS mRNA is significantly up-regulated in the gills of AGD-affected rainbow trout 7 and 14 dpe but not in the liver or anterior kidney (Bridle *et al.* 2006b).

Expression patterns of iNOS can be quantified by qRT-PCR, however the abundance and distribution of the cells expressing the translated protein can not be identified using PCR techniques and may be important for elucidating host cellular interactions with the parasite. It may be surmised that iNOS expressing cells would be predominately associated with gill lesions and *Neoparamoeba* spp. during AGD as the cells that produce iNOS may be associated with bactericidal activity (Maffei, Mirels, Sobel, Clemons & Stevens 2004). We attempted to assess the presence of iNOS bearing cells in the gills of Atlantic salmon using immunohistochemical techniques and antiserum (rabbit anti-rat iNOS antibody AB5382, Chemicon International, Baroma, Australia) previously reported to bind to the putative iNOS molecule of rainbow trout (Barroso, Carreras, Esteban, Peinado, Martinez-Lara, Valderrama, Jimenez, Rodrigo & Lupianez 2000). Results from this study may provide valuable information regarding the role of NO production in destroying *Neoparamoeba* spp. at the site of the infection and also the role that NO production plays in the development of characteristic hyperplastic lesions.

Studies investigating the effect of ectoparasitic infection on fishes immune system response have found increases in EGCs in the affected tissue (Powell, Briand, Wright & Burka 1993; Reite & Evensen 1994; Abraham & Arock 1998; Reite 1998; Dezfuli *et al.* 2000; Buchmann, Sigh, Nielsen & Dalgaard 2001; Saha, Tonkal, Croft & Roy 2004). The exact nature of piscine EGCs is a matter of contention and numerous hypotheses have been proposed. The most common theory is that EGCs are part of the host inflammatory response to infection. This hypothesis is based upon EGC similarities with mammalian mast cell morphology and staining properties (Reite 1998; Reite & Evensen 2006). In mammals, mast cells are involved in inflammatory reactions, cellular growth, leucocyte differentiation and activation. They play a prominent role in the pathology of helminth and other parasitic infections (Weir & Stewart 1997; Marshall 2004). It is also thought mast cells may play a direct role in pathogen killing, mediated by the production of anti-microbial peptides (piscidins) (Silphaduang & Noga 2001; Marshall 2004). Piscidins have been identified in tissue associated mast cells in hybrid striped bass (*Morone saxatilis* × *M. chrysops*) (Silphaduang & Noga 2001). Gill infection of bream (*Abramis brama*) with the crustacean ectoparasite *Ergasilus sieboldi* results in high numbers of EGCs and rodlet cells in the infected primary and secondary lamellae (Dezfuli *et al.* 2003). It was hypothesized that the EGCs played an important inflammatory role in the response of the *E. sieboldi* infected gill epithelium (Dezfuli *et al.* 2003). The presence of EGCs in the liver, pancreas and peritoneal serosa of the minnow (*Phoxinus phoxinus*) parasitized with the nematode *Raphidascaris acus* has been reported (Dezfuli *et al.* 2000). As the EGCs were concentrated around the reactive foci of the infection it was suggested that they play an important role in an integrated inflammatory network

(Dezfuli *et al.* 2000). EGCs have been identified in the gills of AGD-affected Atlantic salmon (Adams & Nowak 2001; Adams & Nowak 2003; Adams & Nowak 2004b), however their role during AGD is unclear and their presence is not characteristic of the disease. Based upon the hypothesized role of EGCs in other parasite infections, their incidence, timing of presence and association with *Neoparamoeba* spp. during infection may be of importance in further understanding the inflammatory events that occur during AGD. Furthermore such information may enhance our knowledge of EGCs as a member of the piscine inflammatory response. The presence of EGCs in Atlantic salmon intestine have been identified using a polyclonal rabbit anti-human lysozyme antibody (EC3.2.117, DakoCytomation Pty Limited, Botany, NSW) which was purported to bind to Atlantic salmon lysozyme within the EGCs (Sveinbjornsson, Olsen & Paulsen 1996). The antibody was therefore used to identify cells in the gills of AGD-affected Atlantic salmon.

The aim of this study was to identify leucocytes that have immunological relevance in parasitic infections, principally EGCs and those cells expressing iNOS or Ig. This was to be achieved using multiple immunohistochemical staining techniques on AGD-affected gill tissue sections to demonstrate the presence and interaction of these immunologically relevant cells. We describe the presence of few Ig bearing cells in the gills of AGD-affected and unaffected Atlantic salmon and discrepancies between the reported cross-reactivity of commercial antisera with Atlantic salmon antigens.

Material and Methods

Fish and experimental induction of *Neoparamoeba* spp. infection

One hundred and fifty Atlantic salmon (*Salmo salar*) (mean weight 100 ± 5 g) from the University of Tasmania Aquaculture Centre were acclimated to filtered (5 µm) sea water (35 ‰ salinity) over a period of 10 d. Fish had been previously kept only in fresh water since their arrival from the Wayatinah hatchery (Saltas Pty Ltd) and therefore had not been exposed to *Neoparamoeba* spp. prior to the commencement of the experiment. Atlantic salmon were maintained in 4000 L Rathburn recirculating systems with separate biofilters and were fed once daily to satiation. One tank of fish were exposed to 100 cells L^{-1} of *Neoparamoeba* spp. using a standard challenge method (Morrison, Crosbie & Nowak 2004) whilst the other tank remained unexposed (uninfected control). Sequential sampling of 5 fish per tank per time point occurred 0, 4, 8, 16 and 32 days post-*Neoparamoeba* spp exposure. (DPE). Fish were randomly removed from each tank, euthanised (clove oil, 0.2 % v v^{-1}), gills excised, and the left gill arches placed into seawater Davidson's (SWD) fixative. Gills were subsequently transferred to 70 % ethanol, dehydrated, embedded in paraffin, sectioned at 5 µm and mounted on superfrost Polysine® glass microscope slides (Menzel, HD Scientific Supplies Pty Ltd, Sunshine, Vic, Australia) for immunohistochemical staining. The proportion of AGD-affected gill filaments was calculated using the method described by Adams & Nowak (2003). AGD-affected filaments (%) = [(The number of correctly orientated filaments with typical AGD lesions / total number of correctly orientated filaments) × 100]. Confirmation that the amoebae seen on the gills of AGD-affected fish were *Neoparamoeba* spp. was performed immunohistochemically using rabbit anti-*Neoparamoeba* spp. antiserum. Gill

sections were processed as described below using the immunohistochemistry (IHC) technique.

Validation of antibodies for immunohistochemical identification of gill leucocytes.

Tissue and serum samples

Serum, liver and anterior kidney were obtained from stimulated Atlantic salmon (AGD-affected) and rainbow trout (injected with *Vibrio anguillarum*), whilst spleen was isolated only from Atlantic salmon. Anterior kidney and liver tissue homogenates were obtained using the same method. The use of either type of tissue was dependent upon the localisation of the antigen of choice (Table 3-1).

Table 3-1 Tissue samples used to characterise antibodies by localisation of specific antigens in the tissue.

Species	Sample	Antibody		
		Anti-Ig	*Anti-iNOS*	*Anti-Lysozyme*
Atlantic salmon	*Serum*	✓	✓	✓
	Anterior kidney	✗	✓	✓
	Liver	✗	✓	✓
	Spleen	✓	✗	✗
Rainbow trout	*Serum*	✗	✓	✓
	Anterior Kidney	✗	✓	✓
	Liver	✗	✓	✓

The anterior kidney and liver were aseptically obtained from euthanised fish (0.2 % clove oil v v^{-1}), and homogenised 1:3 (w v^{-1}) in lysis buffer (30 mM Tris-HCL, 5 µM dithiothreitol, 10 µM EDTA, 15 µM EGTA) (Barroso *et al.* 2000). Homogenates were centrifuged at 16 000 × *g* for 1 h at 4 °C and the supernatants collected. Supernatants were diluted 1:4 in SDS (sodium dodecyl sulphate) reducing sample buffer containing 5 % v v^{-1} β-mercaptoethanol and 95 % stock

sample buffer (1.2 mL 0.5 M Tris-HCl pH 6.8, 1.0 mL glycerol, 2.0 mL 10 % w v^{-1} SDS, 0.5 mL 0.1 % w v^{-1} bromophenol blue, 4.8 mL distilled water), supernatants were boiled for 5 min before being stored at -20 °C for later use. Serum samples were diluted 1:40 in saline (0.9 % w v^{-1} NaCl) before diluting 1:4 in SDS reducing sample buffer. Protein concentrations of the samples were measured using a Pierce BCA protein assay kit (Progen, Darra, Australia).

SDS-PAGE and Western blotting techniques

Polyacrylamide separating gel percentages were chosen based upon the size of the peptide of interest: 12 % - lysozyme, 10 % - immunoglobulin, and 7.5 % - iNOS. Briefly, separating and running gels consisted of separating (75mL 2M Tris-HCl, pH 8.8, 4 mL 10 % w v^{-1} SDS, 21 mL distilled water) or running buffers (50 mL 1M Tris-HCl, pH 6.8, 4 mL 10 % w v^{-1} SDS, 46 mL distilled water), bis acrylamide (Bio-Rad Laboratories Pty Ltd, Regents Park, Australia), sterile distilled water, SDS, ammonium persulphate and TEMED (N,N,N',N'-Tetramethylethylenediamine, Sigma).

Atlantic salmon and rainbow trout reduced liver (0.34 mg $well^{-1}$ total protein), anterior kidney (0.24 mg $well^{-1}$ total protein), and serum (1.0 mg $well^{-1}$ total protein) samples were loaded into respective wells (20 µL sample $well^{-1}$). Positive controls (lysozyme and purified immunoglobulin) and broad-range molecular weight markers (Bio-Rad Laboratories Pty Ltd, Regents Park, Australia) were also loaded into the gel wells (10 µL per well) and gels were electrophoresed (20

mA per gel, approximately 1 h) in electrophoresis buffer (25 mM Tris, 192 mM glycine, 3.5 mM SDS pH 8.8).

Following electrophoresis, gels were stained with Coomassie brilliant blue R-250 (Sigma-Aldrich Pty Ltd) or electrotransferred (200 v, approximately 1 h) onto Hybond™ -C Extra nitrocellulose membrane (Amersham Biosciences Pty Ltd, Castle Hill, Australia) under semi-dry conditions. The nitrocellulose membrane and blotting paper were pre-wetted with transfer buffer (48 mM Tris, 39 mM glycine, 20 % v v^{-1} methanol, 80 % v v^{-1} distilled water, pH 9.2) prior to transfer. Once transferred, membranes were stained with ponceau stain (Sigma-Aldrich Pty Ltd) and the molecular weight marker lane stained with amido black (Sigma-Aldrich Pty Ltd). Non-specific binding sites on the membrane were blocked using 5 % skim milk (SM) powder in tris buffered saline (TBS, 20 mM Tris-HCl, 500 mM NaCl, pH 7.5) for 1 h at room temperature (RT, 18 – 20 °C). Following blocking, membranes were washed in TBS, TBS-tween (TTBS, 0.05 % Tween-20 and TBS), and TBS for 5 min each at RT. Blots were probed with primary antisera or normal rabbit serum (Sigma-Aldrich Pty Ltd) (diluted to an Ig concentration according to the manufacturer recommendations) diluted in 1 % SM-TBS for 1.5 h at RT. Membranes were washed and incubated with the secondary horseradish peroxidase (HRP) conjugated goat anti-rabbit Ig antibody (1:500, 1 h, RT with shaking) (Bio-Rad Laboratories Pty Ltd, Regents Park, Australia). Following incubation with the secondary antibody, membranes were washed and developed using diaminobenzidine (DAB) and urea/hydrogen peroxide (Sigma-Aldrich Pty Ltd).

Optimization of IHC staining methods

Tissue samples for histology were fixed in seawater Davidson's and seawater formalin (one gill arch each), transferred to 70 % ethanol, dehydrated, embedded in paraffin, sectioned at 5 μm and mounted on superfrost Polysine® glass microscope slides for immunohistochemical staining. In order to optimise the conditions for IHC staining heat induced epitope retrieval (HIER) was tested empirically to assess if its use enhanced specific staining. HIER was tested on all tissue samples and both fixatives. Briefly, sections to be HIER were de-waxed, re-hydrated and transferred to 500 mL citrate buffer (10 mM tri-sodium citrate, 10 mM citric acid, pH 6.0), microwaved on high (1300 W) for 12 min and left to stand (20 min) at which time they were briefly rinsed in distilled water. They were then treated as per other sections (described below). Sections were blocked for endogenous peroxidase activity (3 % H_2O_2 – 10 min, RT), washed (PBS - × 2 × 3 min) and incubated with normal goat serum (20 min, RT) (Vector Laboratories Inc, Burlingham, US) to block non-specific binding sites. Sections were then incubated with the primary antisera (according to the manufacturer) or normal rabbit serum (at the same dilution as the primary antisera) in a humid chamber (30 min, 37 °C, diluted in PBS). Sections were washed, incubated (30 min, 37 °C) with biotinylated goat anti-rabbit IgG (ABC kit, Vector Laboratories Inc, Burlingham, US), washed again, then incubated (30 min, RT) with peroxidase conjugated streptavidin (ABC kit, Vector laboratories Inc). After the final washing step, sections were incubated with Vector® *Nova*Red™ substrate kit for peroxidase (Vector Laboratories Inc) for 3 min at RT, rinsed in deionised water, counterstained in Mayer's haematoxylin (10 dips), rinsed, differentiated, dehydrated, cleared and mounted.

Distribution of Ig bearing cells in the gill

Time-trial study of Ig bearing cells

Following immunohistochemical staining of the sections for Ig bearing cells, the sections were examined microscopically and the number of Ig bearing cells counted. The distribution of Ig positive cells within different anatomical areas of the gill was noted. The gill was divided into regions, the central venous sinus (CVS), base and tip of the filament, the filamentous epithelia and middle and tip of the lamellae (Figure 3-1). The regions in which the Ig bearing cell were identified was recorded and the association of Ig bearing cells with AGD lesions and the interaction/association of Ig bearing cells with *Neoparamoeba* spp. was also noted. In AGD-affected fish, Ig bearing cells in both normal and lesion affected filaments were counted. Cells in lesions were counted separately.

Ig bearing cells in interlamellar vesicles

As only a few, small interlamellar vesicles (ILVs) were identified in the sections from this study and few leucocytes were found within them, archived gill material was used to identify Ig bearing cells in ILVs. Archived seawater Davidson's fixed gills from Atlantic salmon exposed to aggressive laboratory infections were obtained and stained as described. A total of 10 individual fish from a previous experiment (Chapter 6), were selected based upon previous histological examination of the gills. Exposure time, proportion of filaments with AGD lesions and method of infection induction are shown. The archived, embedded gills were sectioned, immunostained and the interlamellar vesicles examined for the presence of Ig bearing cells within them (Table 3-2).

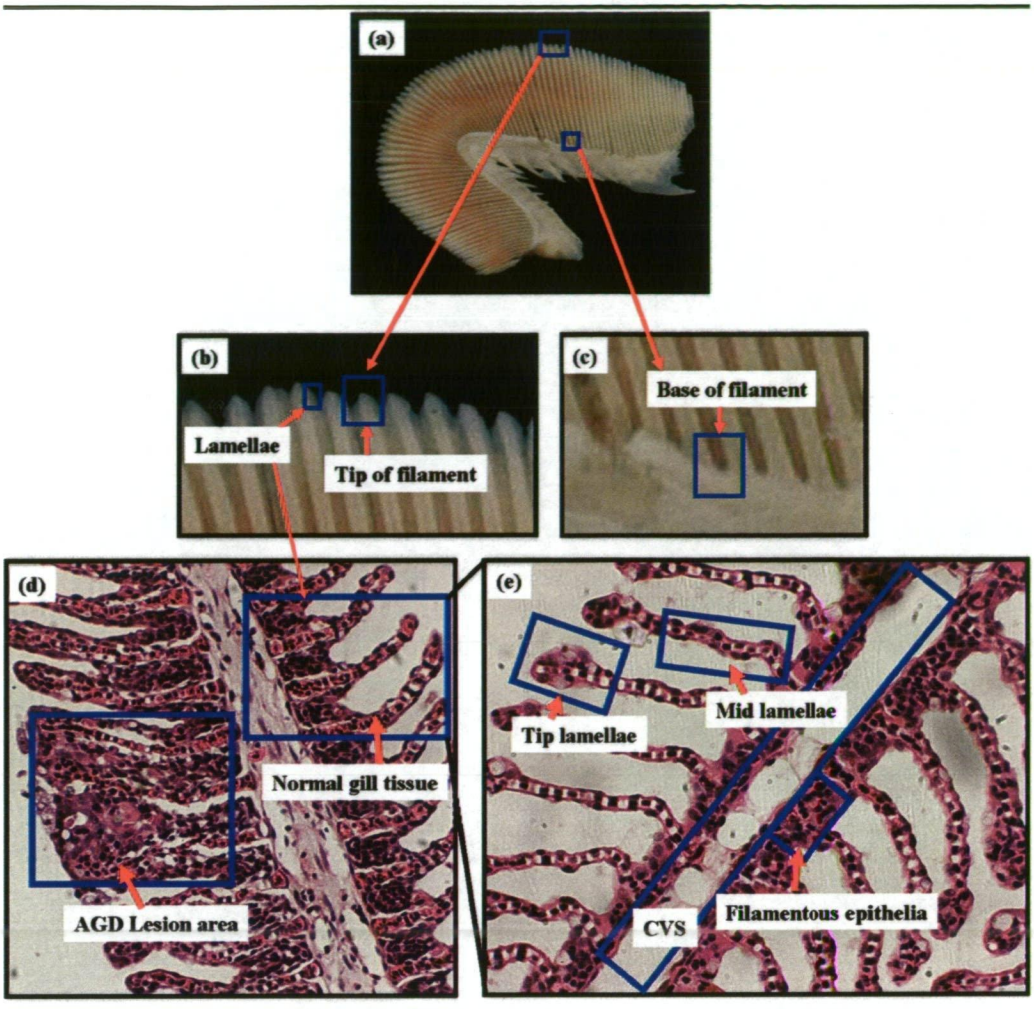

Figure 3-1 Photographs identifying regions of the gills for counting Ig bearing cells. The gill (a) was divided into 2 regions: tip of filament and lamellae (b), base of filament (c). Ig bearing cells within AGD lesion areas were counted separately (d). Normal gill tissue was divided into regions (e): tip and middle of lamellae, CVS (central venous sinus), and filamentous epithelium.

Statistical Analyses

Data are presented as mean ± standard error. Filaments with AGD lesions, number of total Ig bearing cells per filament and the number of Ig bearing cells per tip of filament data were compared using a two-way analysis of variance (ANOVA). Data were tested for normality of distribution using Levene's test for homogeneity, if data were non-homogenous then they were log transformed for analysis. Tukey's test was used to differentiate between means and differences were considered significant at $P < 0.05$ level. All other data were not compared

statistically as there were low number of Ig bearing cells in the other regions of the filament and high variability and statistical analysis may not be reflective of a biological significance.

Table 3-2 Presence of Ig bearing cells in the interlamellar vesicles of the gills from AGD-affected Atlantic salmon.

Fish	Treatment	DPE	Filaments with AGD lesions (%)
1	Horizontal transmission*	5	97
2	Horizontal transmission*	5	84
3	Second time infected (S)^	18	83
4	Second time infected (F)^	18	91
5	Second time infected (F)^	18	66
6	Second time infected (S)^	25	97
7	Second time infected (S)^	25	87
8	Second time infected (S)^	25	75
9	Second time infected (S)^	25	85
10	First infection^	25	68

*Fish infected via horizontal transmission were placed in a tank with AGD-affected fish and unknown density of *Neoparamoeba* spp..

^Fish second time infected had been previously affected with AGD, freshwater bathed and allowed to recover in either freshwater (F) or seawater (S) for 6 weeks before being exposed to 3300 cells L^{-1} *Neoparamoeba* spp., first infection fish were infected in the same tank however had not had previous exposure (Gross, Morrison, Butler & Nowak 2004b).

Results

Validation of antibodies for immunohistochemical identification of gill leucocytes.

Rabbit anti-human lysozyme antibody

Hen egg white lysozyme (HEWL) was used as a positive control during the characterisation of this antibody (rabbit anti-human lysozyme EC3.2.117,

DakoCytomation Pty Limited, Botany, NSW), the minimum amount of lysozyme detectable using this method was < 3.9 μg (data not shown). The rabbit anti-lysozyme bound to HEWL (≈14 kDa) (Figure 3-2). This acted as a positive control for the antibody and the technique. There was evidence of cross-reactivity of the rabbit anti-lysozyme antiserum with higher molecular weight proteins in the ≈ 60-90 kDa range, however there was no evidence of binding to Atlantic salmon lysozyme (≈ 14 kDa) (Figure 3-2). There also appeared to be some binding of normal rabbit serum to a protein in Atlantic salmon serum with an approximate molecular weight of 14 kDa (Figure 3-2). These results are inconsistent with the report by Sveinbjornsson *et al* (1996) that the rabbit anti-lysozyme antibody bound to Atlantic salmon lysozyme.

Rabbit anti- rat iNOS antibody

Atlantic salmon and rainbow trout anterior kidney, liver and serum samples were used to characterise the rabbit anti-rat iNOS antibody (rabbit anti-rat iNOS antibody AB5382, Chemicon International, Baroma, Australia). Western blots containing antigens from these samples were probed with rabbit anti-rat iNOS antibody at a dilution of 1:2000 (manufacturer recommendation) (Figure 3-3).

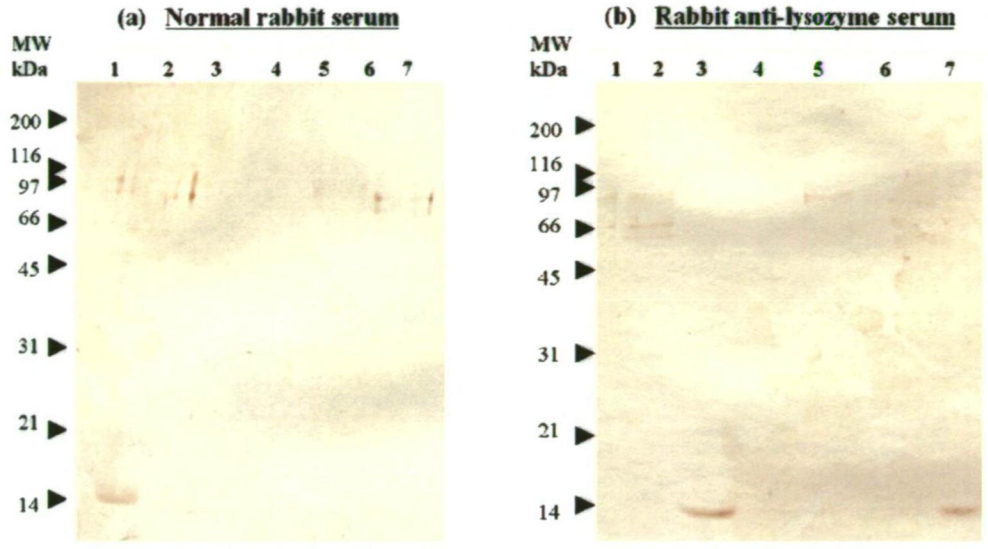

Figure 3-2 Western blot analysis of hen egg white lysozyme (HEWL) (positive control), Atlantic salmon, rainbow trout serum and anterior kidney homogenate probed with normal rabbit serum (1:200) (a) or rabbit anti-lysozyme antiserum (1:200) (b). Membranes were then incubated in HRP conjugated goat anti-rabbit antiserum (1:500) and developed using diaminobenzidine (DAB) and urea/hydrogen peroxide. Lane 1 = Atlantic salmon serum, lane 2 = Atlantic salmon anterior kidney homogenate, lane 3 = HEWL (1.25 μg well^{-1}), lane 4 = blank, lane 5 = rainbow trout serum, lane 6 = rainbow trout anterior kidney homogenate, lane 7 = HEWL (1.25 μg well^{-1}). Arrows indicate position of molecular weight markers (kDa).

There was evidence of binding of the anti-iNOS antibody to proteins isolated from rainbow trout liver and anterior kidney samples. This positive band was at the approximate correct molecular weight (≈130 kDa) (lanes 5 and 6) for iNOS, however significant binding occurred at a protein band with a smaller molecular weight in both Atlantic salmon and rainbow trout samples (≈ 50 kDa). The identity of this protein is unknown.

Rabbit anti-Atlantic salmon immunoglobulin antibody

The rabbit anti-Atlantic salmon immunoglobulin antibody (a gift from Dr Dina Zilberg) (1:2000) was characterised using both purified Atlantic salmon Ig (purified from Atlantic salmon serum using a mannan binding protein affinity chromatography column, Progen Biosciences, Archerfield, Australia) and whole

diluted (1:40) Atlantic salmon serum (Figure 3-4). The antibody bound to both purified Atlantic salmon Ig and serum protein at a molecular weight consistent with the Ig heavy chain (≈ 70 kDa).

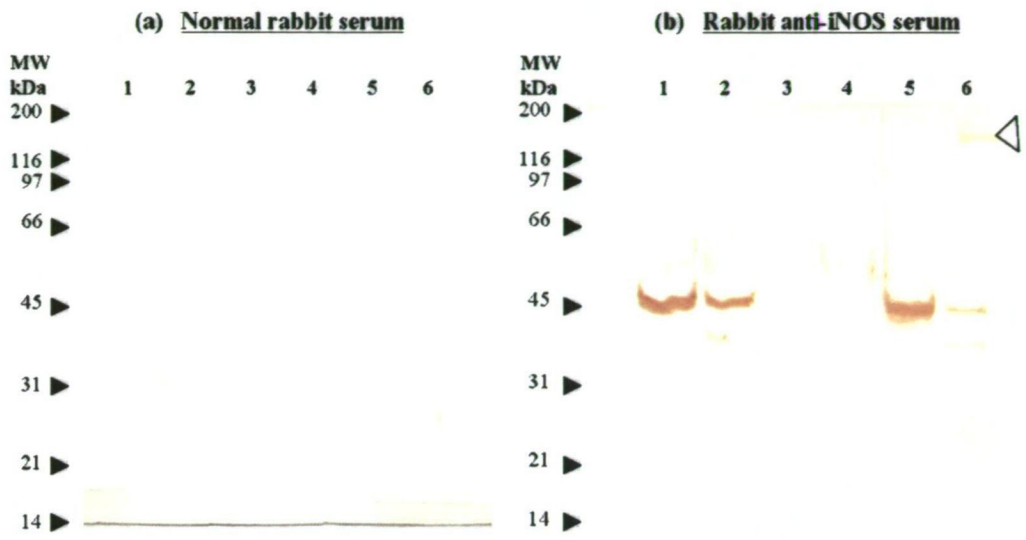

Figure 3-3 Western blot analysis of Atlantic salmon and rainbow trout serum, anterior kidney and liver samples probed with (a) normal rabbit serum (1:2000) or (b) rabbit anti-rat inducible nitric oxide synthase (iNOS) polyclonal antibody (1:2000). Membranes were then incubated in HRP conjugated goat anti-rabbit antiserum (1:500) and developed using diaminobenzidine (DAB) and urea/hydrogen peroxide. Lane 1 = Atlantic salmon serum, lane 2 = Atlantic salmon liver homogenate, lane 3 = Atlantic salmon anterior kidney homogenate, lane 4 = rainbow trout serum, lane 5 = rainbow trout liver homogenate, lane 6 = rainbow trout anterior kidney homogenate. Filled arrows indicate position of molecular weight markers (kDa) and open arrow indicates expected position of iNOS (≈130 kDa).

Optimization of IHC staining methods

Histological sections of Atlantic salmon anterior kidney tissue were probed with anti-lysozyme antibody (1:200) to determine if the antiserum would bind antigens associated with anterior kidney cells. HIER did not enhance antigen identification and the optimal fixative was SWD (Figure 3-5). Atlantic salmon liver was probed with anti-iNOS antibody (1:2000) to identify if any binding would occur that may not have been observed in the Western blot. There were no

iNOS-cross reactive bearing cells identified with or without HIER and with either of the fixatives (example Figure 3-6).

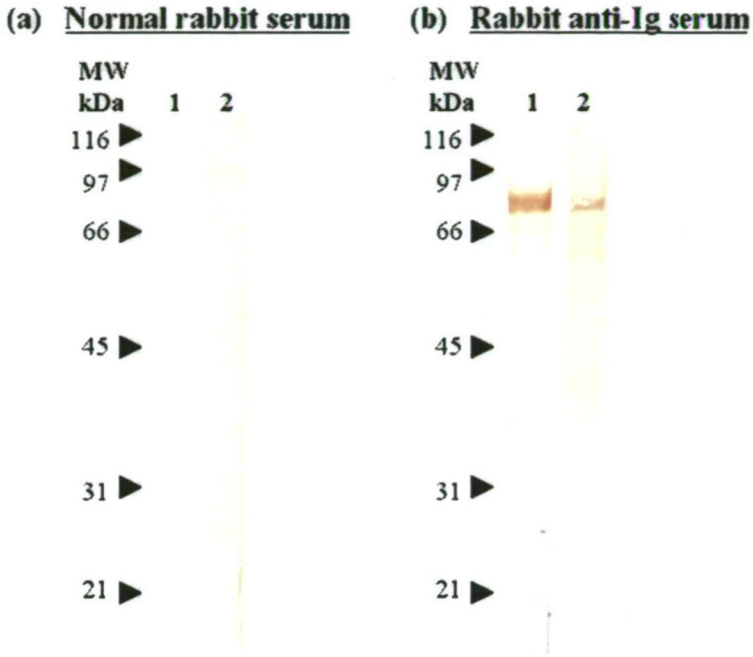

Figure 3-4 Western blot analysis of Atlantic salmon serum and purified Ig probed with (a) normal rabbit serum (1:2000) or (b) rabbit anti-Atlantic salmon Ig serum (1:2000). Membranes were then incubated in HRP conjugated goat anti-rabbit antiserum (1:500) and developed using diaminobenzidine (DAB) and urea/hydrogen peroxide. Lane 1 = Atlantic salmon purified Ig, lane 2 = Atlantic salmon serum. Arrows indicate position of molecular weight markers (kDa).

Spleen tissue sections were used to optimise the IHC staining technique for identification of Ig bearing cells (Figure 3-7). Optimal IHC staining occurred when SWD fixed tissue was used, compared to seawater formalin (SWF) fixed tissue. HIER did not significantly enhance antigen identification but increased background staining (figures not shown). IHC staining technique as described above was used, with the optimal dilution of the rabbit anti-Atlantic salmon immunoglobulin being 1:2000. Once optimal staining of the spleen was achieved this method was trialled with gill tissue to ensure that the conditions that were optimal for spleen tissue were also optimal for gill. The presence of

Neoparamoeba spp. on the gills of AGD-affected fish was confirmed using IHC staining techniques and a rabbit anti-*Neoparamoeba* spp. antibody (1:400) (Figure 3-8).

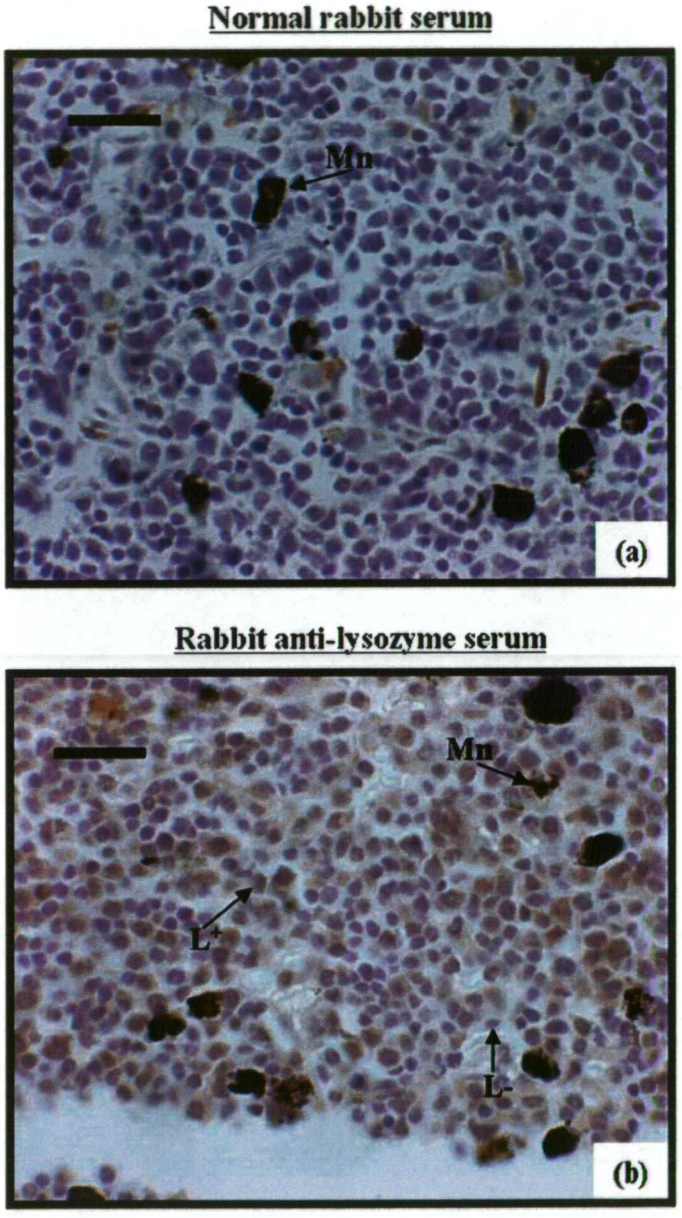

Figure 3-5 Atlantic salmon anterior kidney probed with (a) normal rabbit serum (1:200) and (b) rabbit anti-lysozyme antibody (1:200). Note melanomacrophages (Mn), NovaRed™ stained rabbit anti-lysozyme antibody bearing cells (L^+), anti-rabbit lysozyme non-bearing cells (L^-). Bars = 10 μm.

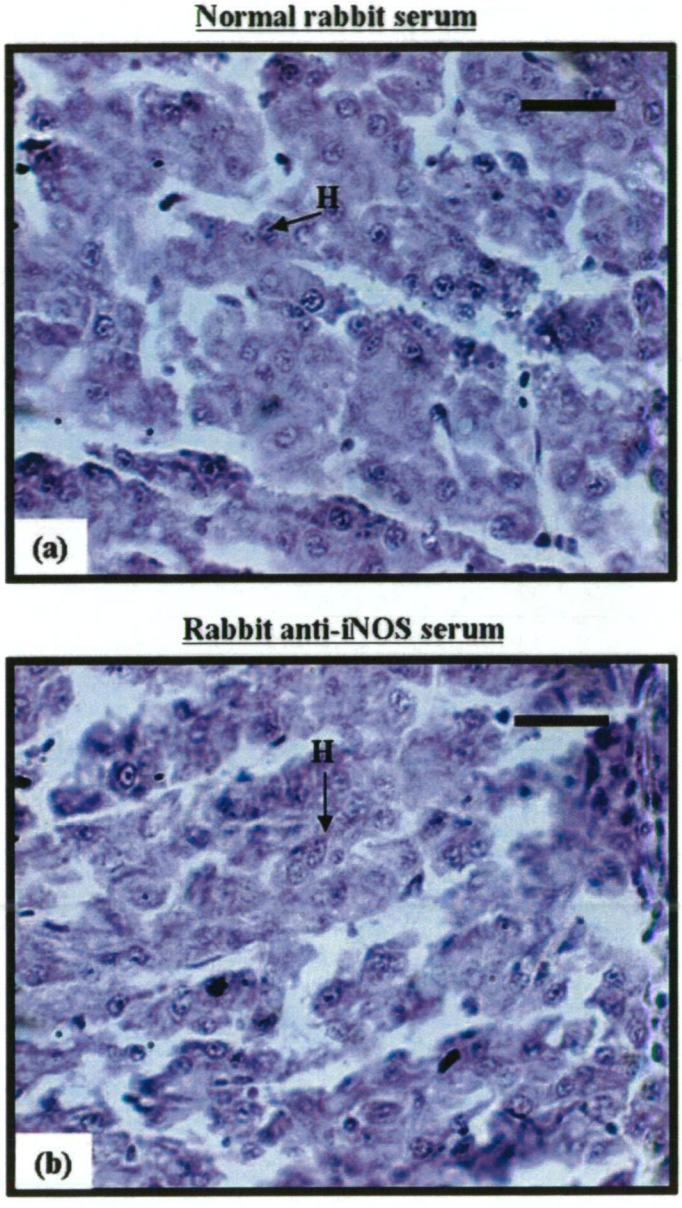

Figure 3-6 Atlantic salmon liver probed with (a) normal rabbit serum (1:2000) and (b) rabbit anti-iNOS antibody (1:2000). Hepatocytes (H). Bars = 10 μm.

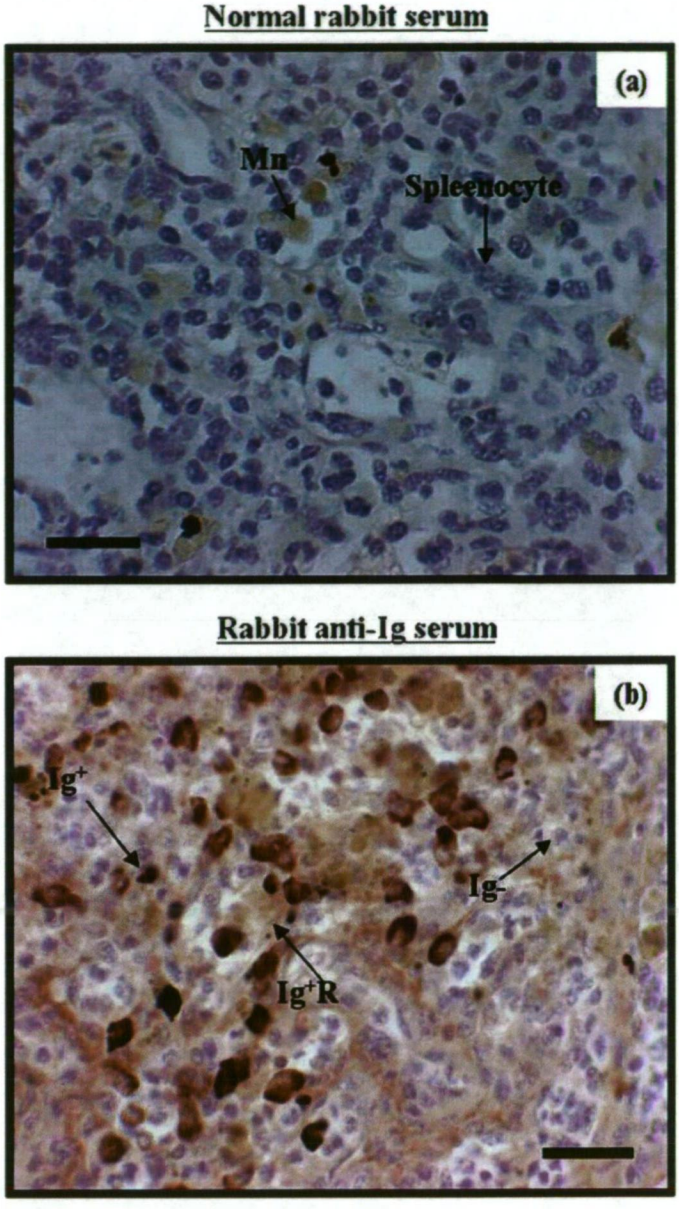

Figure 3-7 Atlantic salmon spleen probed with (a) normal rabbit serum (1:2000) and (b) rabbit anti-Atlantic salmon immunoglobulin serum (1:2000). Note NovaRed™ stained rabbit anti-Atlantic salmon immunoglobulin bearing cells (Ig^+), rabbit anti-Atlantic salmon immunoglobulin negative cells (Ig-), rabbit anti-Atlantic salmon immunoglobulin positive reticulum (Ig^+R), splenocytes and melanomacrophages (Mn). Bars = 10 μm.

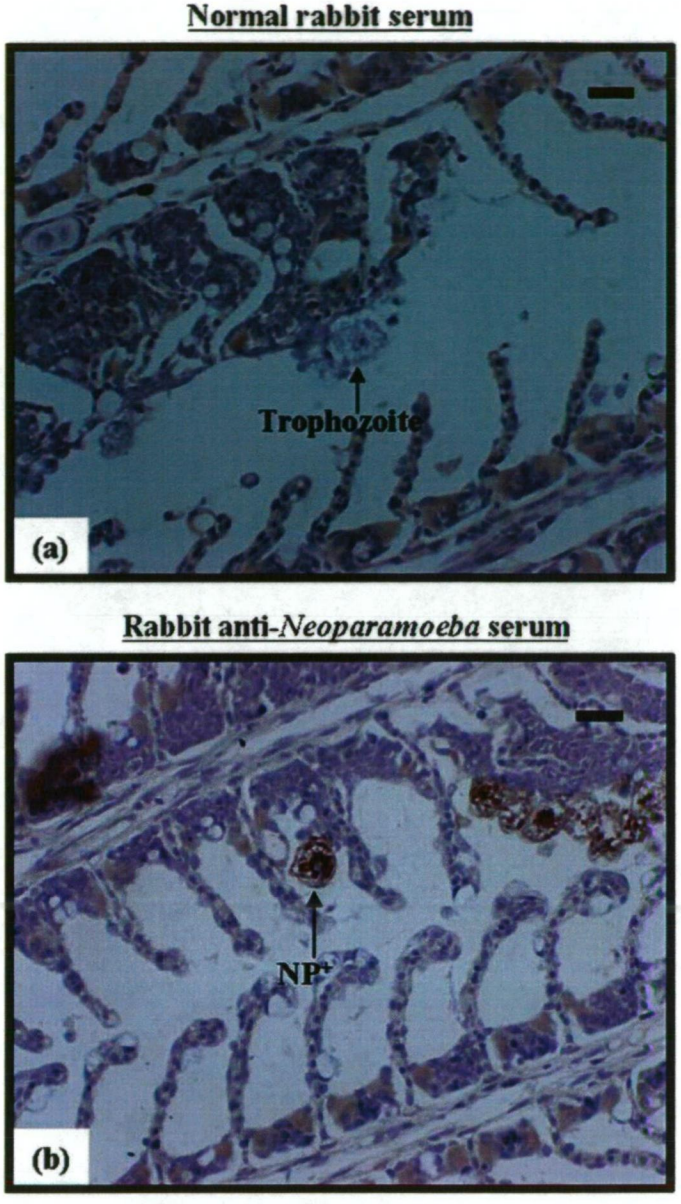

Figure 3-8 Immunohistochemical identification of *Neoparamoeba* spp. on the gills of AGD-affected Atlantic salmon. AGD-affected gills were probed with normal rabbit serum stained (1:400) and rabbit anti- *Neoparamoeba* spp. serum (1:400). Note NovaRed™ stained trophozoite with anti- *Neoparamoeba* spp. antibody bound (NP^+) and unstained trophozoite. Bars = 20 µm.

Distribution of Ig bearing cells in the gill

Time-trial study of Ig bearing cells

Over the 32 d of this trial there were no mortalities in both the AGD-affected and unaffected groups. The proportion of AGD-associated gill lesions in the exposed group closely resembled the severity of those seen in Atlantic salmon culture

situations where AGD is reported (M. Adams personal communication). At no stage were there gross or histological signs of AGD in the control (unexposed) tank of fish. The proportion of filaments affected with AGD lesions 0, 8, 16, 24 and 32 DPE was 0 %, 30.6 % (± 9.0), 29.0 % (± 8.4), 54.4 % (± 5.3) and 59.2 % (7.8) respectively (mean ± standard error) (Figure 3-9). The presence of *Neoparamoeba* spp. on the gills was demonstrated by immunohistochemical identification of *Neoparamoeba* spp. (Figure 3-10).

Ig bearing cells were found in all areas of normal uninfected (control) fish gill (Figure 3-11a-g). The majority of Ig bearing cells were found at the tip of the gill filaments in both the control and infected groups of fish. However, this distribution was not statistically significant ($P > 0.05$) compared to the other regions of the gill. There was no significant ($P > 0.05$) effect of exposure to *Neoparamoeba* spp. on the total number of Ig bearing cells per filament in the gill (Figure 3-12), the number of Ig bearing cells in the tips of filaments (Figure 3-13) or the mean number of Ig bearing cells per filament found in the CVS, base of the filament, filamentous epithelia and the middle or tip of the lamellae (Table 3-3). There were few Ig bearing cells located in AGD lesions and the presence of these cells in the lesions appeared to be co-incidental rather then an active response (Table 3-3, Figure 3-14 a-c).

Ig bearing cells in interlamellar vesicles

There was no evidence of Ig bearing cells in the interlamellar vesicles of the archived samples or the samples from the current study, (Figure 3-14 c).

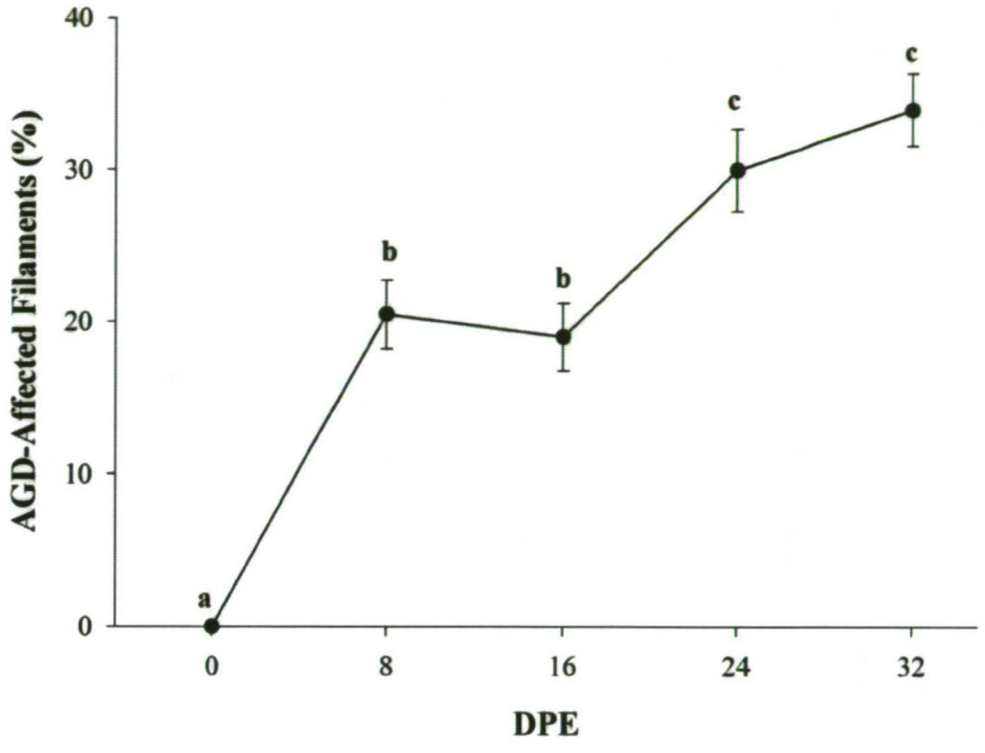

Figure 3-9 The proportion of AGD-affected gill filaments (%) over time after *Neoparamoeba* spp. exposure. Infected = fish exposed to 100 cells L^{-1} of *Neoparamoeba* spp.. DPE = days post *Neoparamoeba* spp. exposure. No AGD-associated lesions were found on the gills of unexposed fish (data not shown). Different letters indicate significant differences ($P < 0.05$) over time, n = 5.

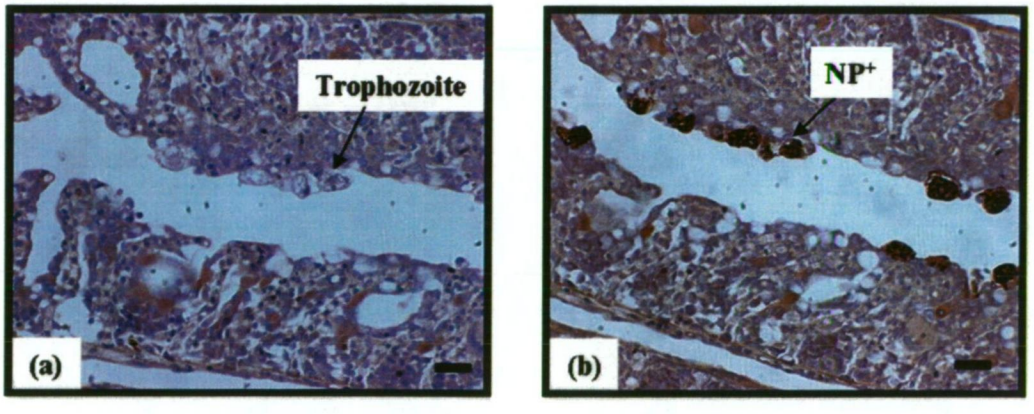

Figure 3-10 AGD-affected Atlantic salmon gill probed with (a) normal rabbit serum (1:200) and (b) rabbit anti-*Neoparamoeba* spp. (1:200) and stained with NovaRed™. Trophozoite unstained with NovaRed™ and (b) NovaRed™ stained *Neoparamoeba* spp. (NP). Bars = 20 µm.

Table 3-3 Mean (± standard error) number of Ig bearing cells per filament of *Neoparamoeba* spp. exposed (infected) and unexposed (control) Atlantic salmon gills. Gills were separated into regions consisting of the CVS (central venous sinus), lesions, filament base and filamentous epithelia, and the middle and tip of the lamellae. NA = not applicable

DPE	CVS		Lesion	Filament				Lamellae			
				Base		Filamentous epithelia		Middle		Tip	
	Infected	*Control*	*Infected*	*Infected*	*Control*	*Infected*	*Control*	*Infected*	*Control*	*Infected*	*Control*
0	1.05	1.50	NA	0.09	0.04	1.19	1.71	0.04	0.04	0.00	0.01
	(.12)	(.38)		(0.02)	(0.04)	(0.81)	(0.51)	(0.04)	(0.02)	(0.00)	(0.01)
8	2.55	1.92	0.16	0.04	0.03	1.84	1.11	0.11	0.03	0.07	0.01
	(0.30)	(0.30)	(0.07)	(0.03)	(0.03)	(0.27)	(0.25)	(0.05)	(0.02)	(0.04)	(0.01)
16	1.39	2.17	0.03	0.00	0.06	1.92	1.46	0.13	0.02	0.03	0.05
	(.29)	(.31)	(0.03)	(0.00)	(0.04)	(0.49)	(0.11)	(0.03)	(0.02)	(0.03)	(0.05
24	2.97	2.57	0.41	0.33	0.09	2.21	2.66	0.12	0.22	0.11	0.26
	(0.44)	(0.22)	(0.22)	(0.2)	(0.03)	(0.53)	(0.98)	(0.04)	(0.13)	(0.05)	(0.11)
32	1.93	2.03	0.09	0.12	0.06	1.82	1.75	0.1	0.08	0.05	0.08
	(0.50)	(.33)	(0.04)	(0.11)	(0.03)	(0.5)	(0.6)	(0.04)	(0.07)	(0.04)	(0.07)

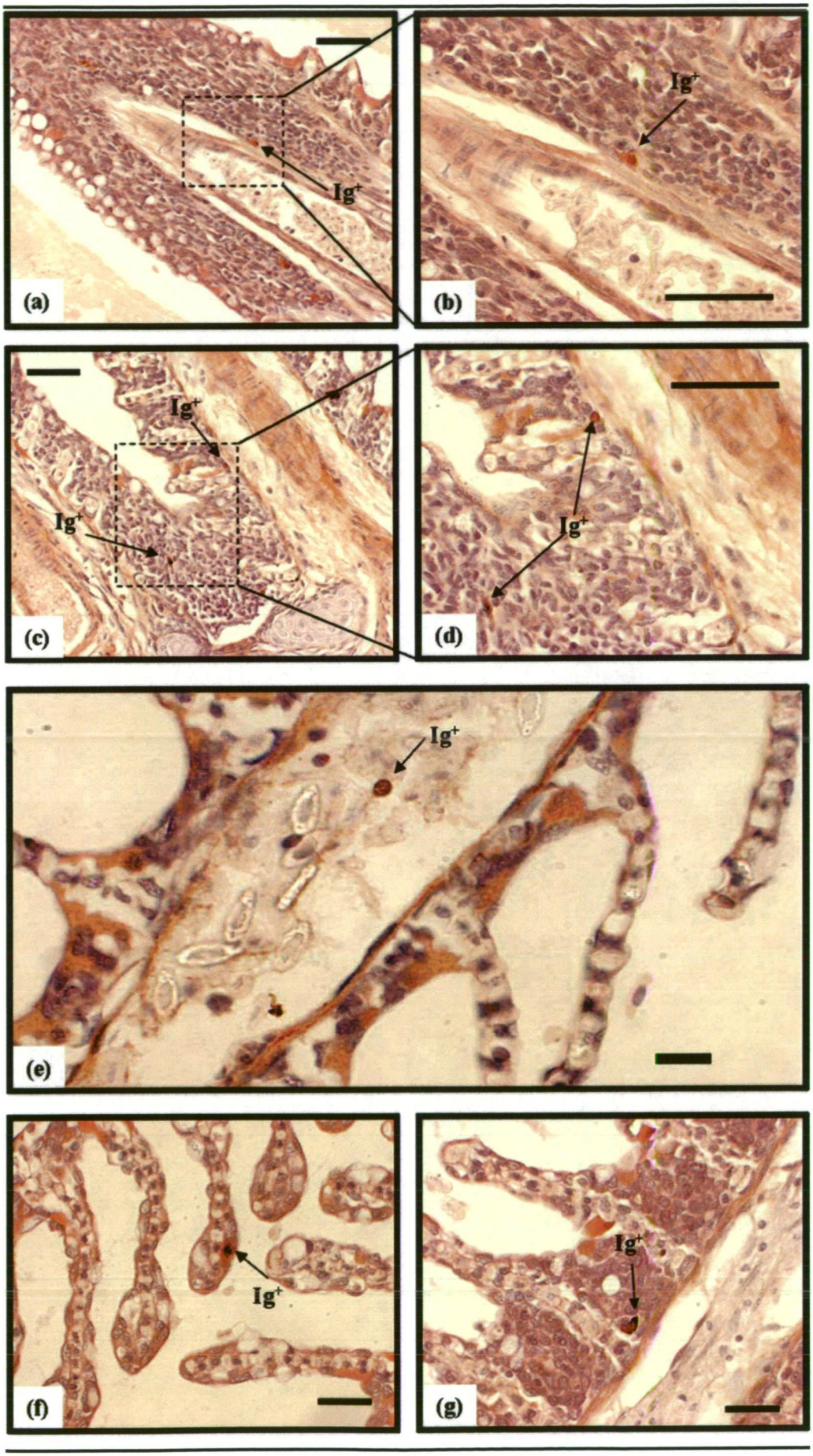
(a)
Ig+
(b)
Ig+
(c)
Ig+
Ig+
(d)
Ig+
(e)
Ig+
(f)
Ig+
(g)
Ig+

Figure 3-11 Normal (unexposed to *Neoparamoeba* spp.) gills probed with rabbit anti-Atlantic salmon Ig serum (1:2000). (a-b) Tip of gill filament with Ig bearing cells (Ig^+), (c-d) base of filament with Ig bearing cells (Ig^+), (e) central venous sinus with Ig bearing cell (Ig^+), (f) tip of secondary lamella with Ig bearing cell (Ig^+), (g) filamentous epithelium with Ig bearing cell (Ig^+) are shown. Square insert in figure (3-11 a) is figure (3-11 b) at lower magnification. Square insert in figure (3-11 c) is figure (3-11 d) at lower magnification (see previous page for figure). Bars = 20 μm.

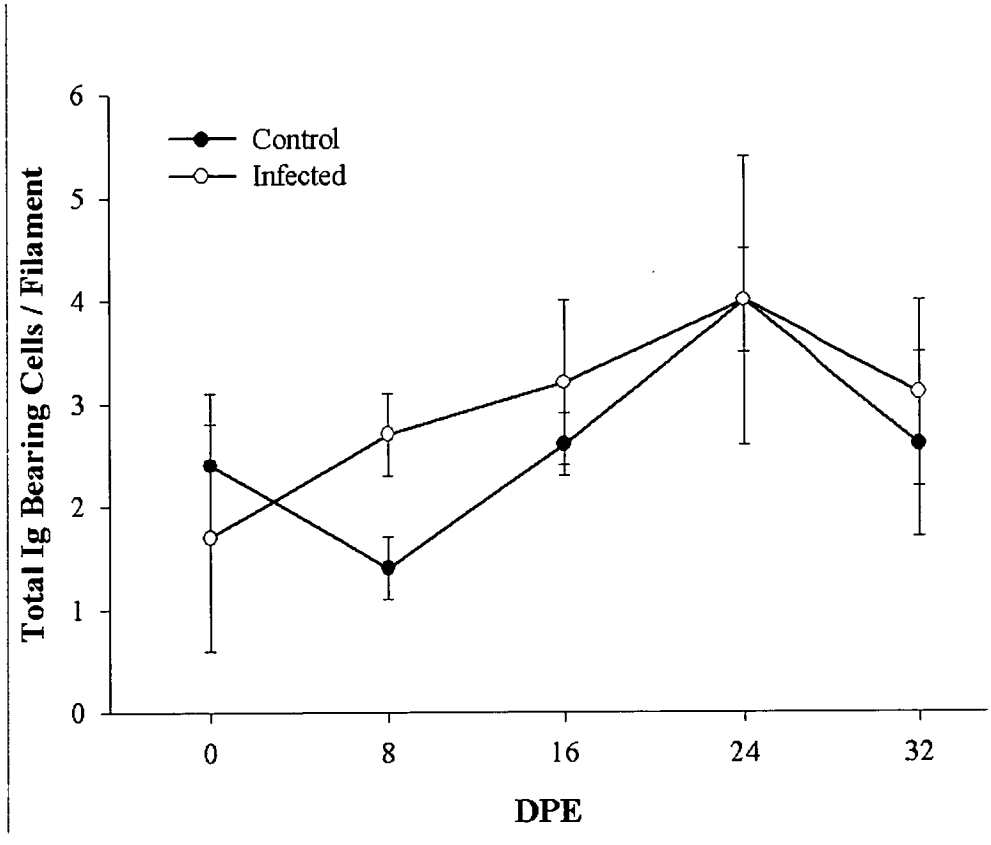

Figure 3-12 Total number of Ig bearing cells per filament. Control = fish unexposed to *Neoparamoeba* spp.. Infected = fish exposed to 100 cells L^{-1} *Neoparamoeba* spp. after day 0 sampling. DPE = days post-*Neoparamoeba* spp. exposure. No significant difference between treatments or over time ($P > 0.05$).

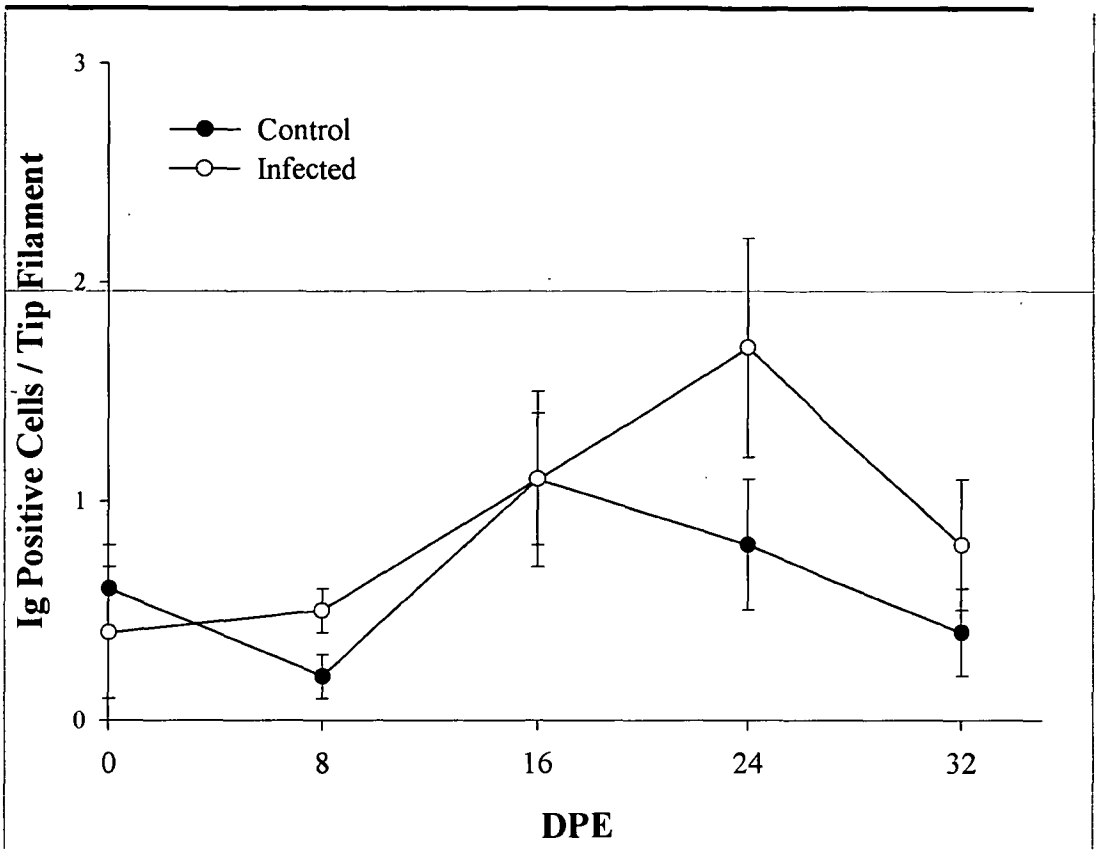

Figure 3-13 Mean number of Ig bearing cells at the tip of each filament. Control = fish not exposed to *Neoparamoeba* spp.. Infected = fish exposed to 100 cells L^{-1} *Neoparamoeba* spp. after day 0 sampling. DPE = days post-*Neoparamoeba* spp. exposure. No significant difference between treatments or over time ($P > 0.05$).

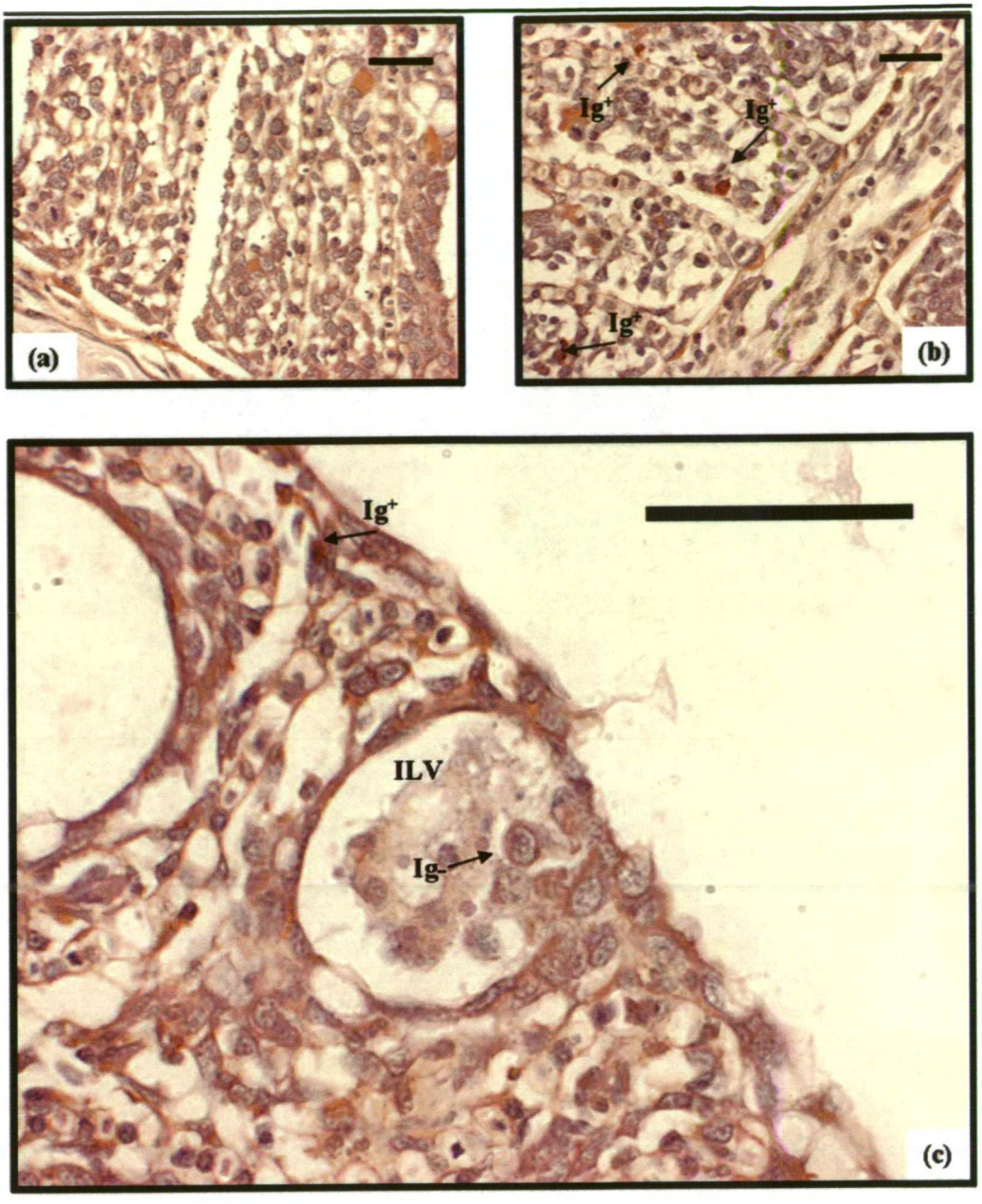

Figure 3-14 AGD-affected Atlantic salmon gill probed with (a – c) rabbit anti-Atlantic salmon Ig serum (1:2000) are shown. Note (a) hyperplastic lesion with no Ig bearing cells, (b) hyperplastic lesion with Ig bearing cells (Ig^+), (c) interlamellar vesicle (ILV) with leucocytes within and no Ig bearing cells, Ig-leucocyte (Ig-) and the presence of Ig bearing cells within the surrounding tissue (Ig^+). Bars = 20 μm.

have direct contact with the pathogen in an environment physiologically suited to the leucocyte rather than the amoebae.

MHC-II expressing cells have been identified in Atlantic salmon spleen, anterior kidney, gill, thymus and the peripheral blood (Koppang, Hordvik, Bjerkas, Torvund, Aune, Thevarajan & Endresen 2003). MHC-II bearing cells have also been identified in the gills of AGD-affected Atlantic salmon (Morrison *et al.* 2005). In mammals MHC-II bearing cells are essential for the presentation of antigens to T cells. T cells in turn proliferate and differentiate into T helper cells which are capable of producing B-cell growth and differentiation factors and ultimately resulting in the production of plasma cells, memory cells and the production of specific antibodies (Weir & Stewart 1997). Similar mechanisms of antigen processing and presentation are thought to occur in teleost fish (Vallejo, Miller & Clem 1992).

Results from the present study show that Ig bearing cells are present in the gills of AGD-affected fish but their numbers do not increase during infection relative to control fish. This suggests that the gill is not a primary site for the production of specific anti-*Neoparamoeba* spp. antibodies. There are many MHC-II cells present in lesions (Morrison *et al.* 2005) but few Ig bearing cells so it is possible that antigen presentation may occur away from the site of the gill. As a consequence, Ig bearing cells producing specific anti-*Neoparamoeba* spp. antibodies may be present in areas such as the anterior kidney and/or spleen but not in the gill. This explains the presence of antibodies in the serum (Howard &

Carson 1995; Gross *et al.* 2004a) and the lack of them in gill mucus (Howard & Carson 1995). In mammals B cells may also express MHC-II molecules (Weir & Stewart 1997). If piscine B cells share these markers it is possible that the MHC-II bearing cells found in lesions may have been B lymphocytes too and therefore it would be expected that they would be found to be Ig bearing. MHC-II bearing cells have been identified in the gill of AGD-affected fish (Morrison et al. 2005) and may be of a number of lineages including macrophages, epithelial cells and lymphocytes (Koppang et al.2003). However, as the results of this present study indicate that there are few B-cells in the gill of AGD-affected fish, the increase in number of MHC-II bearing cells previously identified (Morrison et al. 2005) was probably not due to increases in B-cells as there are few Ig bearing cells in the gill of AGD-affected fish.

The anterior kidney is a site of significant antibody secreting cell generation in fish (Kaattari, Bromage & Kaattari 2005). In rainbow trout it has been demonstrated that upon systemic stimulation the anterior kidney has substantially more antibody secreting cells than the peripheral blood or spleen and that this is concurrent with an increase in the antibody titre of the serum (Kaattari *et al.* 2005). Interestingly, whilst AGD is a localised disease, systemic effects have been found both in anterior kidney cellular immune cell function (Gross *et al.* 2004b; Gross, Powell, Butler, Morrison & Nowak 2005), the production of serum anti-*Neoparamoeba* spp. antibodies (Findlay *et al.* 1995; Akhlaghi *et al.* 1996; Gross *et al.* 2004a) and expression levels of cytokines in the anterior kidney and liver (Bridle, Morrison, Cupit Cunningham & Nowak 2006a; Bridle *et al.* 2006b). Atlantic salmon exposed to *Neoparamoeba* spp. during culture produce a

detectable serum antibody titre specific for *Neoparamoeba* spp. (Gross *et al.* 2004a). This suggests that there is a presentation and processing of the antigen within the fish most likely by MHC-II bearing cells. However, as there is not an abundance of Ig bearing cells in the gills it does not appear that the gill is a major organ for antibody secretion during AGD and that presentation of the antigen to these cells may in fact occur elsewhere in the fish other than the gill. In turbot (*Scophthalmus maximus*) infected with the myxozoan parasite *Enteromyxum scophthalmi* increased numbers of Ig bearing cells were detected in the intestine which is the target organ of the parasite (Bermudez *et al.* 2006). Interestingly, the number of Ig bearing cells initially increased in the hematopoietic organs of the spleen and anterior kidney and then decreased over the course of the infection (Bermudez *et al.* 2006). Increases in Ig bearing cell populations occurred in the hematopoietic areas and subsequently the Ig positive cells migrated to the tissue area affected (intestine). Furthermore the immunosuppression that occurred later in the disease was associated with the decreased numbers of Ig bearing cells in the spleen and kidney (Bermudez *et al.* 2006). The results from the present study do not reflect a similar pattern of host response to parasitic infection as there was not a significant increase in Ig bearing cells in the gills of AGD-affected Atlantic salmon over the course of the infection. It would be worthwhile to investigate the response of the anterior kidney and spleen cell populations of Atlantic salmon affected with AGD. Past studies have demonstrated a decrease in the innate cellular function of anterior kidney cells isolated from AGD-affected Atlantic salmon (Gross *et al.* 2004b; Gross *et al.* 2005).

The presence of leucocytes within interlamellar vesicles of AGD-affected fish gills has been reported on numerous occasions (Roubal *et al.* 1989; Adams & Nowak 2001; Adams & Nowak 2003; Bridle *et al.* 2003; Adams & Nowak 2004a; Adams & Nowak 2004b). However exact leucocyte lineages are yet to be identified and putative identifications have relied upon morphological characteristics of cells. In this study it was found that at no time were any of the leucocytes associated with interlamellar vesicles Ig bearing. Within these vesicles cells morphologically similar to macrophages and neutrophils have been seen interacting with *Neoparamoeba* spp. (Adams & Nowak 2001; Bridle *et al.* 2003). Incidental observations during this study noted that at no time did the *Neoparamoeba* spp. associated with the gills show signs of Ig being bound to their surface, which may have been an indicator of specific anti-*Neoparamoeba* spp. antibody presence in the gill. Immunostaining of Ig around *E. scophthalmi* stages in the intestine of infected turbot may have indicated the presence of specific antibodies generated by the fish in response to the parasite (Bermudez *et al.* 2006).

Unfortunately it was not possible to assess the presence of EGCs or iNOS bearing cells using IHC techniques as the antibodies did not bind to their respective antigens under the conditions tested herein. The rabbit anti-lysozyme antibody bound to proteins in Atlantic salmon serum, anterior kidney homogenates and whole cells as demonstrated by protein bands on the Western blot and IHC stained tissue sections. However the putative molecular weight of the protein to which the antibody bound was not consistent with lysozyme. Presently, there is only a partial sequence available for Atlantic salmon lysozyme (TrEMBL

accession number Q9DD55) and the putative molecular weight of the partial sequence is 12 kDa however its would be expected that it would be greater than this given only a partial sequence has been described. The complete rainbow trout lysozyme sequence is available and based upon the reported amino acid sequence it has an approximate molecular weight of 14.7 kDa (Dautigny, Prager, Pham-Dinh, Jolles, Pakdel, Grinde & Jolles 1991). The partial Atlantic salmon lysozyme sequence was used to interrogate the GenBank database by BLAST (Altschul, Gish, Miller, Myers & Lipman 1990). It had significant identity with the rainbow trout lysozyme sequence (CAA42084) (99/105 residues were the same). Therefore based upon the similarity scores of these two proteins it would be expected that Atlantic salmon lysozyme would have a similar complete sequence and a similar molecular weight. Sveinbjornsson *et al* (1996) reported binding of this rabbit anti-lysozyme antibody to purified rainbow trout lysozyme as demonstrated with an ELISA and also binding to Ig in Atlantic salmon tissue samples, however no Western blot or SDS-PAGE analysis of the purified protein was reported. Perhaps there was binding of rabbit Ig to some other protein in the serum. The purification method used by Sveinbjornsson *et al* (1996) to extract the lysozyme may not have been 100 % effective resulting in residual proteins. Furthermore the Sveinbjornsson *et al* (1996) study purified lysozyme from rainbow trout and used this purified product as the control for the lysozyme detection assay (enzyme linked immunosorbent assay). There is no evidence of any binding between the rabbit anti-lysozyme antibody and rainbow trout lysozyme in the Western blot from this current study. The antibody used in this current study binds to some fraction of rainbow trout and Atlantic salmon serum and anterior kidney isolates and it also binds to an undetermined population of

cells in the anterior kidney. However, proteins that it bound were not consistent with the expected molecular weight of Atlantic salmon lysozyme or in the known region for rainbow trout lysozyme. Consequentially it was decided there was not sufficient evidence to use this antibody to detect putative-EGCs in the gill.

The rabbit anti-iNOS antibody bound to the putative iNOS molecular region of proteins isolated from rainbow trout liver. The antibody did not bind to the appropriate region for Atlantic salmon iNOS. The method (Barroso *et al.* 2000) used to initially characterise the cross-reactivity of the antibody with rainbow trout iNOS was a more sensitive method for detection of antibody binding on Western blots than the colorimetric method used here. There was binding of the rat anti-iNOS antibody to a protein from the rainbow trout liver homogenate as seen on the Western blot. IHC was performed on Atlantic salmon liver samples to identify if the tissue homogenates had sufficient antigen to be detected using the less sensitive method. However as iNOS-bearing cells could not be detected in the liver using IHC staining no further research was performed. A comparison of rat iNOS and rainbow trout iNOS sequences was performed using BLAST (Altschul *et al.* 1990). It was noted that the immunogen portion of the antibody (AB5382) was not found in the rainbow trout iNOS sequence. This may suggest that reactivity of the antibody was not occurring with rainbow trout iNOS but another protein that was of a similar molecular weight as reduced iNOS. If the correct immunogen portion of iNOS is not present in rainbow trout it may also not be present in Atlantic salmon. This would reflect why the antibody did not bind to putative Atlantic salmon iNOS either *in situ* in the liver or by western blot analysis of liver and kidney homogenates.

The results from this study suggest that the gill is not a site of significant Ig bearing cells during amoebic gill disease. As it has been demonstrated that Atlantic salmon are capable of producing a systemic humoral immune response during infection (Gross *et al.* 2004a) and the presence of putative antigen presenting cells (MHC-II bearing) (Morrison *et al.* 2005) within close proximity to lesions it is suggested that the gill is not the primary region for the production of specific anti-*Neoparamoeba* spp. antibodies.

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Chapter 4 *In vitro* interactions between *Neoparamoeba* spp. and salmonid leucocytes; the effect of parasite sonicate on anterior kidney leucocyte function

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Abstract

Sonicated *Neoparamoeba* spp. did not affect the *in vitro* respiratory burst response of leucocytes isolated from Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhynchus mykiss* Walbaum) and Chinook salmon (*Oncorhynchus tshawytscha* Walbaum) anterior kidneys ($P > 0.05$). However, Atlantic salmon and Chinook salmon leucocytes pre-incubated with the parasites responded to PMA stimulation with a greater response compared to cells incubated with PMA on its own ($P < 0.05$). Sonicated *Neoparamoeba* spp. was not chemoattractive for anterior kidney leucocytes isolated from all three fish species.

Keywords

Neoparamoeba spp., leucocyte, respiratory burst, chemotaxis, phagocyte function, amoebic gill disease.

Amoebic gill disease (AGD) is a significant ectoparasitic disease affecting the gills of mariculture fish in South Eastern Tasmania, Australia and is associated with the protozoan parasite *Neoparamoeba pemaquidensis* (Munday *et al.*, 2001). Recently, *N. branchiphila* have been isolated from AGD-affected fish gills however whether the disease is of mixed origin is unknown (Dyková *et al.*, 2005).

Our understanding of the innate immune response of fish to ectoparasitic infections is limited. Aside from the protection afforded by mucus production, research has focused on bio-active substances such as complement and lysozyme (Jones, 2001). Also, the epidermal migration of inflammatory cells, their secretions and macrophages involved in localized inflammatory reactions are areas of significant research (Jones, 2001). Macrophages are one of the most active leucocytes in the innate immune system and are especially important in defending against the early stages of parasitic infections (Wiegertjes & Joerink, 2004). Because of the central role of macrophages in immunity, it is hypothesised that anterior kidney innate immune cells may play a critical role in the susceptibility or resistance of salmonids to AGD. The aim of this study was to determine if sonicated pathogenic *N. pemaquidensis* and *N. branchiphila* are capable of eliciting an *in vitro* respiratory burst response by anterior kidney leucocytes (innate immune cells) isolated from salmonids.

Due to the potential presence of both *N. pemaquidensis* and *N. branchiphila* on the gills of AGD-affected fish the parasite isolate may contain both of these species and will therefore be referred to as a *Neoparamoeba* spp. (Nspp) isolate. The sonicated Nspp suspension was prepared by isolating gill associated Nspp

from Atlantic salmon (*Salmo salar* L.) using a plastic adherence method (Morrison *et al.*, 2004). Identification of the cells as *Neoparamoeba* spp. was confirmed using immunocytochemistry on whole cells (Bridle *et al.*, 2003). Nspp were counted using 0.25% trypan blue in seawater and fixed with 0.05% formalin (v v^{-1}). The cells were resuspended to a final concentration of 5×10^5 cells ml^{-1} in phenol red-free Hank's balanced salt solution (HBSS) (Invitrogen Australia Pty Ltd, Mulgrave, Australia) and sonicated (×3 pulses, 30 s each, 4 Amp microns). The final suspension was observed microscopically to ensure that the cells had ruptured.

To assess the effect of sonicated Nspp on the *in vitro* generation of reactive oxygen species (ROS), anterior kidney leucocytes were isolated from three salmonid species and incubated with three concentrations of the parasite. These cells were isolated from Atlantic salmon (40.2 ± 12.7 g), Chinook salmon (*Oncorhynchus tshawytscha* Walbaum, 71.3 ± 22.6 g) and rainbow trout (*Oncorhynchus mykiss* Walbaum, 344.2 ± 108.9 g) anterior kidneys using the method outlined by Alcorn et al. (2002). Fish were obtained from the Western Fisheries Research Centre, Seattle, Washington, USA maintained at 12° C in sand-filtered, UV-treated lake water and fed *ad libitum* with commercial feed. Fish were naïve to seawater and therefore they had no prior exposure to *Neoparamoeba* spp. as they are obligate marine organisms. Due to limitations in leucocyte cell numbers, five-fish pools of anterior kidney leucocytes were prepared from each species of fish.

Chinook salmon leucocytes were used to determine the incubation time at which exposure of the leucocytes to the Nspp sonicate would give maximal chemiluminescence (CL) response. Leucocytes were exposed to HBSS or HBSS containing the Nspp sonicate concentrations and the basal CL response was determined as described below. Cells were then incubated for 3, 6 or 12 h prior to the addition of phorbol myristate acetate (PMA) and measurement of the stimulated CL response, as described below. Of the three time points tested optimal stimulation occurred 12 h post-*Neoparamoeba* spp. incubation and therefore this incubation time was used in all subsequent CL experiments (data not shown).

Anterior kidney leucocytes isolated from each species were resuspended to 5×10^6 cells ml^{-1} in HBSS (Life Technologies, Grand Island, U.S.A) containing luminol (1 mg ml^{-1}) (Sigma Aldrich Pty ltd, St Louis, U.S.A). One hundred microlitres of each leucocyte suspension was placed into 32 wells (four columns) of an opaque-white flat-bottom 96-well microplate. Eight wells of each leucocyte preparation each received 100 µl of either HBSS or HBSS containing 5×10^5, 2×10^5 or 5×10^4 sonicated Nspp ml^{-1}. The plate was immediately placed in a luminometer (Lab Systems) at 15° C. The CL responses of triplicate wells of each treatment of each leucocyte preparation were measured for 2 sec every 3 min for 180 min (basal CL response). Leucocytes with or without Nspp were incubated for 12 h after which 20 µl of phorbol 12-myristate 13-acetate (PMA, 2.5 µg ml^{-1} in HBSS) (Sigma Aldrich Pty Ltd) was added to triplicate wells of each treatment. The CL responses of the cells exposed to PMA, and the CL response of duplicate

wells of each treatment without PMA were measured for 2 sec every 3 min for 180 min (stimulated CL response).

The total amount of light produced by the cells in each well during the CL response was measured using a luminometer. For the CL produced by cells measured during the basal CL response, the total light values were used for statistical analysis. For the CL response of cells measured after the incubation step, the total light value of the leucocytes exposed to PMA was corrected by subtraction of the mean total light value of the corresponding leucocytes not exposed to PMA and the resulting values were used for statistical analysis.

For the assessment of chemotaxis, leucocyte pools from each fish species were resuspended at 1.6×10^7 cells ml^{-1} in 3 ml HBSS containing Calcein AM (5 µM) (Molecular Probes Inc, Eugene, U.S.A), and incubated for 1 h at 15° C. The cells were washed 3× for 10 min each in HBSS and resuspended in 3 ml HBSS. For each leucocyte preparation, triplicate lower wells of a Chemo TX 96-well chemotaxis chamber (Neuro Probe, MD, USA) were loaded with 29 µl of either HBSS, HBSS containing 0.5% (v v^{-1}) Chinook salmon serum previously stimulated with zymosan (directional migration control) (Sigma Aldrich Pty Ltd), HBSS containing 0, 5×10^5, 2×10^5 or 5×10^4 sonicated Nspp ml^{-1} or 25 µl of the leucocyte suspension. The chemotaxis chambers were assembled by placing the filter assembly over the lower wells. Next, 25 µl of the leucocyte suspension was placed on the hydrophilic regions of the filter assembly corresponding to all of the lower wells, except the blank and total fluorescence wells. The unused portion of the leucocyte preparation was incubated along with the chemotaxis

plate for 2 h at 15° C. Non-migrated cells were removed from the filter assembly of the chemotaxis plate by twice washing with phosphate buffered saline (PBS) and scraping with a cell scraper. The unused portion of the leucocyte suspension was centrifuged at 500 × *g* for 10 min and the supernatant was diluted with an equal volume of HBSS. Twenty five microliters of the diluted supernatant was injected into three lower chambers of the chemotaxis plate by puncturing the filter membrane with a needle (leaked fluorescence control). The fluorescence of the cells in the lower wells was measured using a luminometer (Lab Systems) with filters for 485 nm excitation and 527 nm emission wavelengths.

For the chemotaxis assay, wells loaded with the live stained cells represented the total fluorescence of all cells (total fluorescence). The wells injected with the diluted unused leucocyte supernatant represented the amount of the Calcein AM dye that leaked from cells and diffused into the lower chamber (leaked fluorescence). The fluorescence of the cells that migrated into the lower wells containing the HBSS represented random migration while the migration of cells in response to the Nspp sonicate represented directed migration. The proportion of cells that traversed the membrane was used for statistical analysis and was determined by the equation:

Maximal Movement (%) = [(test migration fluorescence – leaked migration fluorescence) / (total fluorescence – leaked fluorescence)] × 100.

One-way analysis of variance (ANOVA) followed by Tukey's HSD (honestly significantly different) tests were used to compare treatments within species. All

data were tested for homogeneity of variance using Levene's test and log (ln) transformed if found to be non-homogenous. Differences were considered significant at the $P < 0.05$ level and data are presented as mean ± standard error of mean (SEM). If despite transformation data were still non-homogenous differences were considered significant at the $P < 0.01$ level. As the mass of the rainbow trout was greater than both the Atlantic salmon and Chinook salmon ($P < 0.01$), no comparison of the immunological results between the salmonid species were performed.

The chemiluminescence data were found to be non-homogenous and therefore transformed to create homogeneity. The chemiluminescence responses of anterior kidney leucocytes isolated from Atlantic salmon, Chinook salmon and rainbow trout were not significantly ($P > 0.01$) affected by 12 h incubation with the three concentrations of Nspp sonicates compared to leucocytes incubated with culture media only (Figure 4-1).

Pre-incubation of leucocytes isolated from Atlantic and Chinook salmon with all three Nspp sonicate concentrations for 12 h prior to PMA stimulation resulted in a CL response significantly ($P < 0.01$) greater than leucocytes incubated with the Nspp sonicate only (Figure 4-1). At the highest concentration of Nspp the CL response was greater than PMA stimulation alone ($P < 0.01$) suggesting that at the high concentration of Nspp, a priming effect was elicited. Priming occurs when an agonist that does not invoke a measurable response on its own at a low concentration enhances the response of a subsequent activating agent (Bass *et al.*, 1987; Neufert *et al.*, 2001). Substances such as cytokines and microbial agents

prime neutrophils for enhanced ROS production stimulated or initiated by agents such as PMA (Meyer *et al.*, 1991; Neufert *et al.*, 2001). PMA stimulates the respiratory burst by activation of the protein kinase C (PKC) pathway (MacDougal *et al.*, 1999).

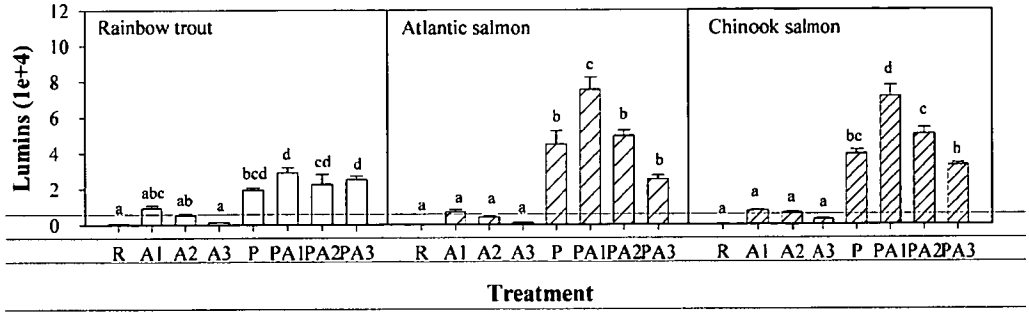

Figure 4-1 Chemiluminescent response of anterior kidney leucocytes isolated from rainbow trout, Atlantic salmon and Chinook salmon exposed to 3 different concentrations of Nspp sonicate for 12 h. (R) = cells were incubated in culture media only for 12 h, (A1) = cells were incubated with 5×10^5 *Neoparamoeba* spp. cells ml^{-1} for 12 h, (A2) = cells were incubated with 2×10^5 *Neoparamoeba* spp. cells ml^{-1} for 12 h, (A3) = cells were incubated with 5×10^4 *Neoparamoeba* spp. cells ml^{-1} for 12 h, (P) = cells were incubated with culture media for 12 h and then stimulated with PMA (2.5 μg ml^{-1}), (PA1) = cells were incubated with 5×10^5 *Neoparamoeba* spp. cells ml^{-1} for 12 h followed by PMA (2.5 μg ml^{-1}) stimulation, (PA2) = cells were incubated with 2×10^5 *Neoparamoeba* spp. cells ml^{-1} for 12 h followed by PMA (2.5 μg ml^{-1}) stimulation, (PA3) = cells were incubated with 5×10^4 *Neoparamoeba* spp. cells ml^{-1} for 12 h followed by PMA (2.5 μg ml^{-1}) stimulation. Different letters indicate significant effect ($P < 0.01$) of treatment on CL response within species.

The mechanisms of priming in mammalian cells are poorly understood. Evidence suggests that enhancement of intracellular signal transduction mechanisms, up-regulation of receptors to activating agents and/or preliminary assembly of membrane-bound and cytosolic components that produce ROS such as NADPH oxidase may occur (Allen *et al.*, 1992; Dahlgren & Karlsson, 1999). The priming response is more effective if each of the agonists interact with different membrane receptors and therefore elicit different intracellular events (McPhail *et al.*, 1984). In the present study incubation of anterior kidney leucocytes with sonicated Nspp

did not induce a measurable response, but when the cells were stimulated with PMA there was a significant increase in their ROS production. Therefore it appears that Nspp are unable to directly stimulate ROS production via either the PKC pathway or by directly activating other mitogen-activated protein kinases. However, cells were primed so as to result in a significantly greater PMA-stimulated response than if stimulated with PMA alone. It appears that whilst there is no statistically significant effect of the Nspp sonicate on leucocyte CL response there is evidence to suggest that if higher doses of Nspp had been used there may have been a dose dependent activation of the cells in all three fish species. Perhaps with a greater concentration of Nspp there may be a significant activation of the basal respiratory burst response following stimulation. Similar studies with macrophages from both fish and mammals have shown that immune cell function may be primed by prior incubation with a pathogen (Verburg-van Kemenade *et al.*, 1996; Neufert *et al.*, 2001; Scharsack *et al.*, 2003). Speculatively, these results indicate that virulence factors of Nspp may be related to their ability to avoid direct host cell stimulation of ROS production. This hypothesis is further supported by data that suggest NADPH-oxidase activity is down-regulated in the gills of Atlantic salmon eight days post infection with *Neoparamoeba* sp. (Morrison *et al.*, 2006).

Rainbow trout leucocytes did not produce a CL response pattern similar to those produced by Atlantic salmon and Chinook salmon leucocytes in response to Nspp incubation. There was no significant difference in the CL response of cells incubated without Nspp and those with Nspp ($P > 0.01$), nor did incubation with Nspp result in a significantly higher CL response when cells were subsequently

incubated with PMA ($P > 0.01$) (Figure 4-1). During AGD infection, rainbow trout have more diffuse mucoid branchitis than the patchy lesions seen in Atlantic salmon (Munday *et al.*, 1990). Also, rainbow trout recovering from AGD have more obvious mononuclear nodules along the primary lamellae than Atlantic salmon (Munday *et al.*, 1990). Examination of histological material from AGD affected Atlantic salmon from Australia appeared identical to that of amoebic infected coho salmon (*Oncorhynchus kisutch* Walbaum) from Washington, USA (Kent *et al.*, 1988). In New Zealand, where significant numbers and varieties of salmonids are cultured under environmental conditions similar to those of Atlantic salmon culture in Tasmania, only a few minor outbreaks of AGD in Chinook salmon have been reported (Munday *et al.*, 2001). The variability in susceptibility to AGD between salmonid species may be due to differences in the innate immune response of the hosts. As outlined in this study there appears to be a difference in the manner by which species respond to this parasite *in vitro*. Whether these *in vitro* differences correspond to *in vivo* dissimilarities in susceptibility is as yet unknown.

The chemotaxis data were found to be non-heterogenous and therefore they were transformed. Migration of leucocytes isolated from Chinook salmon, Atlantic salmon and rainbow trout towards all three concentrations of the parasite sonicate was not different from the random movement control ($P > 0.05$) (Table 4-1). This indicates that leucocytes did not move towards the parasite sonicate, nor did they move away from it. Migration towards the directional movement control was significantly ($P < 0.01$) different from random movement control for leucocytes isolated from both the Chinook and Atlantic salmon but not cells isolated from

rainbow trout ($P > 0.05$). Rainbow trout chemotaxis data have large standard errors in the directional movement control, perhaps this prevented the detection of a statistical difference between the directional movement and the random movement. Optimisation of the chemotaxis method using Chinook salmon leucocytes may have resulted in the assay not being optimal for rainbow trout cells and therefore the stimulation of directional movement could be sub-optimal for this species.

Table 4-1 Chemotactic responses of rainbow trout, Atlantic salmon and Chinook salmon anterior kidney leucocytes to 3 concentrations of Nspp sonicate and the directional migration of the same cells. The values represent the mean ($\pm$ SEM) percentage of the total cell fluorescence. Different superscripts denote significant differences within species ($P < 0.01$).

Species	**% of Maximal Movement**				
	Directional Migration (Zymosan stimulated serum)	*Random movement* (PBS)	*Sonicated Nspp Concentration* (cells ml^{-1})		
			(5×10^5)	(2×10^5)	(5×10^4)
Rainbow trout	42.0^a (7.8)	25.7^a (1.4)	30.2^a (6.1)	25.6^a (4.1)	28.9^a (4.1)
Atlantic salmon	30.5^a (3.7)	17.9^b (0.8)	15.9^b (1.4)	15.8^b (1.1)	15.8^b (1.4)
Chinook salmon	42.9^a (5.3)	28.0^b (1.8)	22.4^b (3.7)	17.8^b (1.1)	21.4^b (2.6)

There was no significant directional movement towards or away from the parasite sonicate by the leucocytes isolated from the anterior kidney of Atlantic salmon, Chinook salmon or rainbow trout. The presence of leucocytes in the central venous sinus of AGD affected fish gills is well documented (Munday *et al.*, 1990; Adams & Nowak, 2001; Adams & Nowak, 2003; Adams & Nowak, 2004a; 2004b). The results from this study suggest that attraction of leucocytes to the site of infection may initially occur as a result of cytokine/chemokine production by localized host cells (e.g. epithelial cells) and not directly as a result of parasite

presence or secretion of extracellular substances. However, it should be highlighted that fixation and/or sonication of Nspp may damage, mask and/or alter the chemoattractive receptors on the parasites cell surface, and therefore further studies using different parasite preparations, alternate concentrations, fractionation studies and other culture supernatants are warranted.

Comparison of phagocyte populations isolated from different fish tissues have shown that the activity of the phagocyte is related to the tissue from which it is taken (Vazzana *et al.*, 2003). For example, leucocytes isolated from the peritoneal cavity of sea bass (*Dicentrarchus labrax*) were found to produce the highest chemiluminescence when stimulated with zymosan, followed by anterior kidney cells, spleen and finally peripheral blood leucocytes produced the least (Vazzana *et al.*, 2003). Furthermore, neutrophils from areas of inflammation have higher phagocytic responses (Matsuyama & Iida, 1999) than those isolated from unaffected areas. We propose that if phagocyte function were assessed in gill leucocytes isolated from AGD affected fish, the response of the cells may in fact be of a greater magnitude than those leucocytes isolated from the anterior kidney. This hypothesis is currently under investigation in our laboratory.

The results from the present study indicate that sonicated Nspp are not capable of directly stimulating salmonid anterior kidney leucocyte production of ROS. However a high concentration of Nspp is capable of priming anterior kidney leucocytes isolated from Chinook salmon and Atlantic salmon for increased ROS production when subsequently stimulated with PMA. When the parasite is fixed and sonicated it does not stimulate nor inhibit directional chemotactic movement

for these leucocytes, another factor which may indicate pathogenicity is related to avoiding host cell stimulation.

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Chapter 5 Changes in the innate immune response of Atlantic salmon (*Salmo salar* L.) exposed to experimental infection with *Neoparamoeba* sp.

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Abstract

An experiment was conducted to determine the effect of *Neoparamoeba* sp. infection on Atlantic salmon innate immune responses. Atlantic salmon were experimentally infected with *Neoparamoeba* sp. and serially sampled 0, 1, 4, 6, 8 and 11 days post-exposure (DPE). Histological analysis of infected fish gill arches identified the presence of characteristic AGD lesions as early as 1 DPE with a steady increase in the number of affected gill filaments over time. Immune parameters investigated were anterior kidney phagocyte function (respiratory burst, chemotaxis and phagocytosis) and total plasma protein and lysozyme. In comparison with non-exposed control fish basal respiratory burst responses were suppressed 8 and 11 DPE, phorbol myristate acetate (PMA) stimulated activity was significantly suppressed 11 DPE. Variable differences in phagocytic activity and phagocytic rate following infection were identified. There was an increase in the chemotactic response of anterior kidney macrophages isolated from exposed fish relative to control unexposed fish 8 DPE. Total protein and lysozyme levels were not affected by *Neoparamoeba* sp. exposure. Together, these results reflect the variable manner in which *Neoparamoeba* sp. infection affects the innate immune response of Atlantic salmon.

Keywords

Amoebic gill disease, Atlantic salmon, *Neoparamoeba* sp., phagocyte function, respiratory burst, *Salmo salar*.

Introduction

The protozoan parasite *Neoparamoeba pemaquidensis* is associated with amoebic gill disease (AGD) a condition which if untreated may cause significant mortalities in sea-cage reared Atlantic salmon (*Salmo salar* L.) in South Eastern Tasmania, Australia (Munday, Zilberg & Findlay 2001). The same parasite has been implicated in AGD in Atlantic salmon and other species of fish throughout the world, including Ireland, Spain, Chile and the USA (Dykova & Novoa 2001). On-farm the presence of white multifocal patches on the gills and an excess of mucus is used to presumptively diagnose AGD and these patches are generally associated with the histopathology of AGD (Adams, Ellard & Nowak 2004).

Host-parasite interactions associated with AGD are not yet fully understood. Moreover characterisation of the mechanisms by which this disease affects the localised and systemic immune response is limited. The local inflammatory response at the site of *Neoparamoeba* sp. infection is characterised by an infiltration of leucocytes at the site of parasite colonisation, hyperplastic epithelial cells (Munday, Foster, Roubal & Lester 1990; Adams & Nowak 2001; Adams & Nowak 2003; Adams & Nowak 2004a; Adams & Nowak 2004b) and increases in mucous cells (Nowak & Munday 1994; Zilberg & Munday 2000; Adams & Nowak 2003; Roberts & Powell 2003).

Previous studies by the authors investigating if fish infected with *Neoparamoeba* sp. and allowed to fully recover in freshwater are resistant to subsequent infection also investigated innate immune parameters. It was demonstrated that fish previously infected with *Neoparamoeba* sp. had suppressed phagocyte function

19 and 26 days post re-infection (Gross, Morrison, Butler & Nowak 2004). However, the previous study was designed to assess the basis of reported (Findlay, Helders, Munday & Gurney 1995; Findlay & Munday 1998; Findlay, Zilberg & Munday 2000) resistance of fish to re-infection following full recovery in freshwater. This resistance was speculated to be due to stimulation of the innate immune response, consequently the effect of *Neoparamoeba* sp. on unexposed fish was not investigated. In the present study, a time-course trial was designed to assess the effect of *Neoparamoeba* sp. exposure on Atlantic salmon systemic immune defences, specifically innate immune cell function (respiratory burst, phagocytic function, and chemotactic ability of anterior kidney phagocytes) and total plasma protein and plasma lysozyme values.

Materials and Methods

Fish

Atlantic salmon (127.6 $\pm$ 33.3 g) smolts were obtained from a commercial salmon hatchery, Wayatinah, Tasmania (Saltas, Salmon Enterprises Tasmania) and maintained in freshwater at the University of Tasmania, Launceston prior to seawater (35 ‰, 1 μm filtered) acclimation over a period of 7 d in 2 × 3000 L recirculation systems (3 × 1000 L tanks per system) connected to individual biofilters. Fish were fed daily to satiation on a commercial food pellet (Skretting, Hobart, Australia).

Inoculation of tank with *Neoparamoeba* sp. and sampling methods

Following acclimation, one system was inoculated with 3300 cells L^{-1} of *Neoparamoeba* sp. as described (Zilberg & Munday 2001). Five fish from each group (5 exposed and 5 unexposed fish) were randomly sampled at 0, 1, 4, 6, 8

and 11 days post-*Neoparamoeba* sp. exposure (DPE). Fish were euthanised in Aqui-S (Aqui-S, NZ Ltd, Lower Hutt, New Zealand) and heparinised blood samples taken from the caudal vein, anterior kidneys were removed aseptically and placed in meshing media comprising Leibovitz-15 medium (L-15) (Invitrogen Australia Pty Ltd, Mulgrave, Australia) supplemented with 2 % FCS (Invitrogen Australia Pty Ltd, Mulgrave, Australia), 0.1 % heparin (10 000 U mL^{-1}) (Sigma-Aldrich Pty Ltd, Castle Hill, Australia), and 1 % penicillin / streptomycin (P / S) (10 000 U mL^{-1}) (Sigma-Aldrich Pty Ltd, Castle Hill, Australia). Anterior kidney tissue samples were kept on ice until required. Gills were removed and placed in seawater Davidson's fixative for routine histology.

Plasma protein and lysozyme

Total plasma protein was measured using a Pierce BCA protein assay kit (Progen, Darra, Australia). Lysozyme was analysed using a 96-well microtitre plate adaptation of the *Micrococcus lysodeikticus* (*M. luteus*) turbidimetric assay (Parry, Chandon & Shahani 1965; Hutchinson & Manning 1996).

Phagocyte isolation

Standard phagocyte isolation techniques were performed (Secombes 1990). Briefly, tissue from the anterior kidney was macerated through an 80 μm mesh using meshing media and the cell suspension placed onto a discontinuous 34 / 51 % Percoll (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) gradients. The cell preparations were centrifuged at 400 × *g* for 35 min, at 4 °C. The cells recovered from the 34 / 51 % interface were washed 3 × with L-15 washing media (0.1 % FCS, 0.1 % heparin, 1% P / S), 400 × *g*, for 10 min, at 4 °C, and counted using a haemocytometer and trypan blue to assess viability. Cell concentrations were

adjusted to 1×10^7 cells mL^{-1} and 100 µL of this suspension was incubated in either 96-well, flat-bottom tissue culture plates (Sarstedt Australia Pty Ltd, Technology Park, Australia) (respiratory burst) or 16 well chamber slides (Medos Company, Waverly, Australia) (phagocytosis) overnight at 18 °C. An aliquot of cells was left gently shaking on a cell-rocker overnight at 4 °C before chemotactic ability was assessed the following day.

Respiratory Burst

The generation of O_2^- by phagocytes was determined by the reduction of nitroblue tetrazolium (NBT) (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) (Secombes 1990). Briefly, phagocyte monolayers were incubated at 18°C for 60 min in 100 µL of NBT solution (1 mg mL^{-1} Hank's Balanced Salt Solution) (HBSS) (Invitrogen Australia Pty Ltd, Mulgrave, Australia) with and without PMA (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) (1 µg mL^{-1} HBBS). Wells were also incubated with NBT, PMA and superoxide dismutase (SOD) (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) (300 µg mL^{-1} HBSS) as controls. Following incubation the reaction was stopped with 80 % methanol, then the cells were washed and allowed to air dry before the addition of 140 µL DMSO (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) and 120 µL 2 M KOH (Sigma-Aldrich Pty Ltd, Castle Hill, Australia). The absorbance was read at 620 nm using DMSO / KOH as the blank. As a method of standardisation independent wells were used in which medium was removed from the wells and the adherent cells lysed using 100 µL of lysis buffer (0.1 M citric acid, 1 % Tween 20 and 0.05 % crystal violet) (Chung & Secombes 1987). The number of phagocytes per treatment was

enumerated by counting the number of released nuclei in a haemocytometer and the absorbance adjusted accordingly.

Phagocytosis

The phagocytosis method was based upon described methods (Thompson, Lilley, Chen, Adams & Richards 1999; Polonio, Wolke, MacLean & Sperry 2000). Briefly, phagocyte monolayers were washed to remove non-adherent cells and then 100 µL 1×10^7 Congo red stained yeast cells (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) were added. The cells were incubated for 60 min at 18 °C at which time non-phagocytosed yeast cells were removed by gentle washing in PBS, the slides were fixed with 80 % methanol and stained with Quick-Dip (Histo Labs, Riverstone, Australia). Cells were examined by light microscopy using magnification 1000 ×. Phagocytic rate (PR) was evaluated by estimating the mean proportion of phagocytes containing at least one yeast cell, in a random count of 100 phagocytes performed in duplicate. The phagocytic index (PI) was determined by estimating the mean number of yeasts within each phagocyte.

Chemotaxis

Chemotaxis was measured using a 48-well micro-chemotaxis chamber (Neuro Probe, Maryland, USA) with a 5 µm pore size polycarbonate filter (Neuro Probe, Maryland, USA) (Falk, Goodwin & Leonard 1980). In the bottom chamber 25 µL of 10 % FCS in PBS (to assess directional movement) or 25 µL of PBS (control for random movement) was placed in the wells and the chamber assembled. The upper-wells were filled with 2.5×10^5 phagocytes (50 µL volume) per well (4 wells per fish, duplicate PBS and 10 % FCS treatments). The chamber was covered and incubated at 18 °C for 60 min. Filters were removed

and the non-migrated cells gently washed off with PBS, the filters were fixed with 80 % methanol and stained in Quick-Dip. The number of migrated cells per field of view (FOV) in at least 10 random fields on the bottom surface of the filter was counted using a light microscope at magnification 1000 ×. Migration ratio was determined as the number of cells FOV^{-1} exhibiting directional movement / number of cells FOV^{-1} exhibiting random movement.

Data analysis

Results from the control uninfected fish and infected fish were analysed using a t test (with degrees of freedom taken into consideration), comparing results between the two groups on sample days. Differences were considered significant at the $P < 0.05$ level. Data were transformed (LN) to create homogeneity of variance where required. Data are displayed as mean $\pm$ standard error of means (SEM).

Results

Neoparamoeba sp. exposed fish began to present very faint white, raised, mucoid patches on their gills 6 DPE, the number and size of the gill patches increased 8 DPE and very heavy gill patches were present 9, 10 and 11 DPE. Mortalities in the AGD affected tanks began 9 DPE and significant mortalities occurred 9, 10 DPE and 11 DPE at which time the trial was terminated. No mortalities occurred in the negative control tanks.

There were no significant differences between the control and infected fish 0 DPE ($P > 0.05$) for respiratory burst, chemotaxis, phagocytosis, lysozyme and total

protein levels. Both the PMA stimulated (11 DPE) and unstimulated (8 and 11 DPE) respiratory burst response of phagocytes isolated from AGD affected fish were significantly decreased compared to phagocytes isolated from unexposed control fish on the respective days (Figure 5-1). In addition stimulation indices (PMA stimulated response / unstimulated response) (Figure 5-1) were significantly different compared to control fish 8 DPE. Chemotaxis results are only available for 0, 6 and 8 DPE, due to limitations in cell numbers. Chemotactic migration ratio (Figure 5-2) was significantly ($P < 0.05$) affected 8 DPE.

Changes in the phagocytic rate and index of anterior kidney phagocytes from infected fish compared to uninfected control fish were variably affected over time (Table 5-1). Total plasma protein and plasma lysozyme remained comparable ($P > 0.05$) between the infected and uninfected control fish throughout the experiment (Table 5-1).

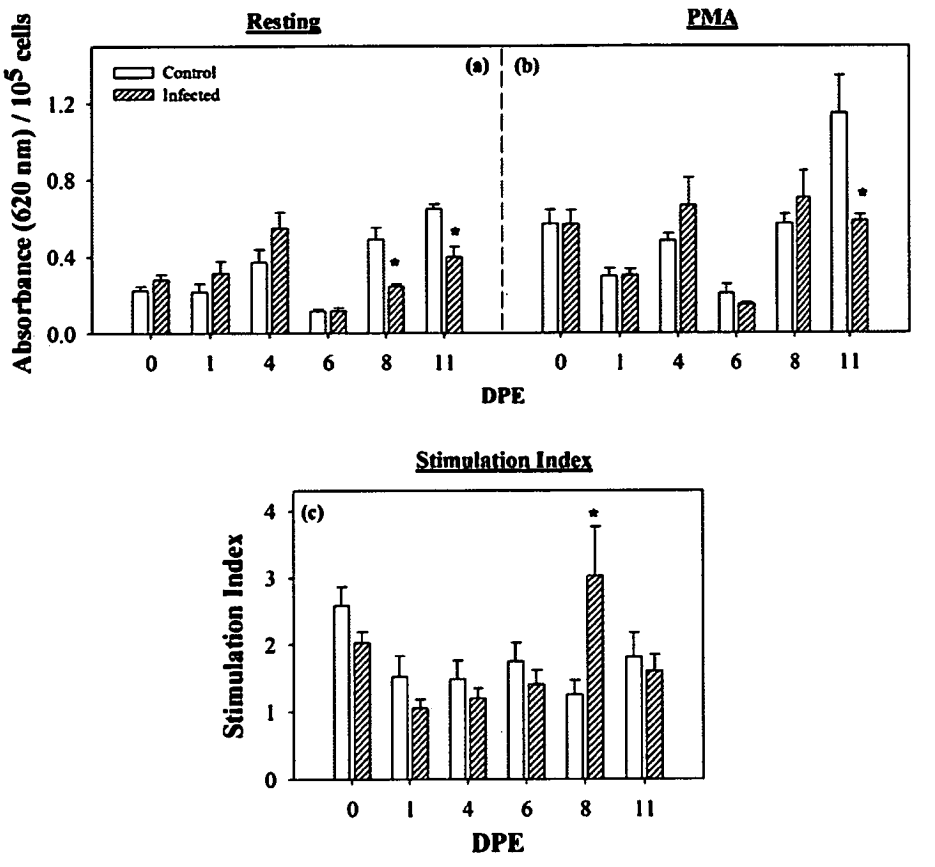

Figure 5-1 The effect of *Neoparamoeba* sp. exposure on basal (a) and PMA (b) stimulated respiratory burst activity and stimulation index (basal respiratory burst activity / PMA stimulated respiratory burst activity) (c) of phagocytes isolated from the anterior kidney of exposed (infected) and unexposed (control) Atlantic salmon. (DPE) days post *Neoparamoeba* sp. exposure. (*) Significantly different from control uninfected fish analysed on the same day ($P < 0.05$), n = 5 fish/group/day.

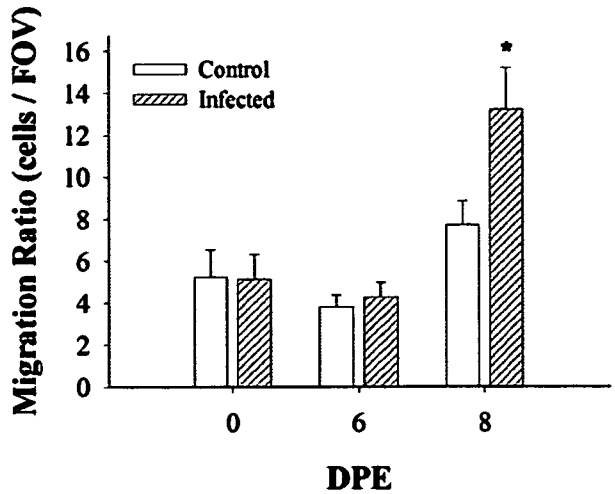

Figure 5-2 The effect of *Neoparamoeba* sp. exposure on migration ratio (directional movement / random movement) of phagocytes isolated from the anterior kidney of exposed (infected) and unexposed (control) Atlantic salmon. (DPE) = days post *Neoparamoeba* sp. exposure. (*) Significantly different result to control uninfected fish ($P < 0.05$), n = 5 fish/group/day.

Table 5-1 The effect of *Neoparamoeba* sp. exposure on phagocytic rate (PR), phagocytic index (PI), plasma lysozyme and total plasma protein of Atlantic salmon. Exposed (infected) and unexposed (control) Atlantic salmon, (DPE) days post *Neoparamoeba* sp. exposure, values presented as mean and MSE, * indicates result significantly different from control ($P < 0.05$), n = 5 fish/group/day.

DPE	PR (%)		PI		Lysozyme (μg ml^{-1})		Plasma Protein (mg ml^{-1})	
	Control	*Infected*	*Control*	*Infected*	*Control*	*Infected*	*Control*	*Infected*
0	65.8 (0.7)	63.5 (5.0)	2.1 (0.1)	2.1 (0.1)	5.3 (1.4)	4.4 (0.8)	40.8 (1.9)	35.2 (3.0)
1	58.1 (2.1)	49.8 * (1.1)	2.0 (0.1)	1.7 (0.1)	7.9 (0.5)	6.6 (0.6)	41.8 (1.8)	37.3 (3.1)
4	58.0 (2.1)	68.3 (3.5)	2.2 (0.2)	2.7* (0.1)	4.7 (0.8)	2.3 (0.8)	39.4 (2.3)	31.9 (3.4)
6	61.6 (2.3)	67.8 * (1.7)	2.4 (0.1)	2.4 (0.0)	2.5 (0.4)	3.8 (0.6)	40.7 (3.0)	40.3 (3.8)
8	57.3 (3.8)	52.0 (5.3)	2.5 (0.2)	2.3 (0.1)	4.0 (1.0)	5.3 (0.5)	48.7 (2.8)	50.9 (2.2)
11	48.9 (2.7)	58.8 * (3.1)	2.2 (0.1)	2.1 (0.1)	3.6 (1.2)	5.3 (0.5)	42.8 (2.4)	45.6 (1.2)

Discussion

In the present study there was a decrease in basal respiratory burst response and an increase in the chemotactic ability of phagocytes isolated from infected fish compared to uninfected fish 8 DPE. However, phagocytes from infected fish could respond equally when the respiratory burst was stimulated with PMA, consequently there was a greater relative response (stimulation index). Neutrophils of mammals such as humans and mice have a respiratory burst response time that is inversely proportional to the strength of the initial disturbance, so as to allow the neutrophils to migrate closer to the source of the signals before producing the respiratory burst (Lehrer & Cohen 1981). A population of primed cells is produced whereby the basal levels may remain the same or lower, however, when the cell is stimulated the relative burst may be increased. In the present study this priming effect appeared to occur concurrently with mortalities and severe gill pathology (8 DPE). During this study characteristic AGD lesions increased over time in the exposed group and there were no clinical or histological signs of AGD in the control unexposed fish (M. Powell, data not shown). It is possible that at this stage of AGD where gill lesions became severe the first significant systemic stimulation occurred as a result of *Neoparamoeba* sp. attachment causing downstream effects on the systemic immune response. This may result in a population of primed phagocytes with apparently suppressed basal levels; equal PMA stimulated respiratory burst responses and therefore an increased stimulation index.

The respiratory burst stimulation index of AGD affected fish was comparable to uninfected fish on all days except 8 DPE. The activity of basal and PMA

stimulated phagocyte respiratory burst was lower in *Neoparamoeba* sp. infected fish than uninfected fish 11 DPE. However, as the stimulation indices were equivalent between the groups it suggests that the relative ability of the phagocytes isolated from the infected fish to respond to stimulation was equal to the uninfected fish and that the PMA stimulated response is probably related to the initial basal response. Deactivation of the respiratory burst response of primed anterior kidney macrophages by sequential *in vitro* stimulation has been demonstrated in fish (Neumann & Belosevic 1996). The respiratory burst response of AGD affected fish may be suppressed due to desensitization of respiratory burst receptors as a result of long-term indirect stimulation by *Neoparamoeba* sp. Due to desensitization of the respiratory burst response their ability to respond to the *Neoparamoeba* sp. on the gills may be inhibited. The present results are supported by previous data demonstrating suppressed respiratory burst results (basal and PMA stimulated) and equal stimulation indices of fish over a long term (26 days) re-infection (Gross *et al.* 2004).

Histological findings have demonstrated an increased number of immune cells at the site of *Neoparamoeba* sp. infection (Adams & Nowak 2003; Adams *et al.* 2004; Adams & Nowak 2004a; Adams & Nowak 2004b). Presumably these leucocytes have been recruited from other leucocyte populations such as the peripheral blood, anterior kidney or spleen. In AGD affected fish it is probable that the decrease in anterior kidney activity is a result of hemopoietic tissue supplying the gill, and intermediately the blood, with more mature and active phagocytes. Furthermore, the increase in chemotactic activity of the phagocytes may have occurred as these cells were in an increased state of chemotactic

movement. Analysis of gill phagocyte behaviour will identify if the site of phagocyte residence influences its activity. This forms part of an on-going study by the authors. In both carp and tilapia (*Oreochromis niloticus*) injected with *Escherichia coli* into the swim bladder, neutrophils found at the site of inflammation had significantly higher phagocytic activity than peripheral blood neutrophils (Matsuyama & Iida 1999). Peripheral blood neutrophils isolated from eels (*Anguilla japonica*) intraperitoneally injected with *Edwardsiella tarda* cells had significantly greater phagocytic rates and chemiluminescence response than those injected with saline, the peripheral neutrophils were also more active than those from the anterior kidney. It was concluded that the anterior kidney supplied the peripheral blood system (site of inflammation) with more mature and active neutrophils in response to the bacterial stimulation (Park & Wakabayashi 1992).

There was a variable, but generally elevated, phagocytic rate of phagocytes isolated from AGD affected fish anterior kidney. The capacity of these phagocytes remained comparable on most days suggesting a stimulation of phagocytes that resulted in an increased number of active phagocytes but not an increased ability to phagocytose.

Non-specific humoral factors were not affected by *Neoparamoeba* sp exposure. Lysozyme is an important anti-bacterial enzyme produced by leucocytes, especially monocytes, macrophages and neutrophils (Yildirim, Lim, Wan & Klesius 2003). As plasma lysozyme levels are regulated by peripheral blood leucocytes (Murray & Fletcher 1976) *Neoparamoeba* sp. infection may not stimulate these cells to produce increased amounts of lysozyme in the serum.

Analysis of mucus lysozyme levels or anterior kidney phagocyte lysozyme production may indicate other mechanisms of lysozyme regulation during AGD. Plasma protein levels were not altered over the course of the infection. Increased vascular permeability resulting in changes in plasma protein values may only occur due to systemic infections and consequently systemic alterations may not be seen as an effect of a localised gill infection. Also, alterations in plasma protein levels may not always be reflected in total protein values (Gerwick, Steinhauer, La Patra, Sandell, Ortuno, Hajiseyedjavadi & Bayne 2002). Furthermore, other innate humoral factors such as complement warrant investigation as complement may play a significant role in immunity towards ectoparasites in fish (Buchmann 1999). Rainbow trout complement *in vitro* binds to and kills the monogenean parasite *Gyrodactylus derjavini* (Buchmann 1998). A pilot study by the authors did not find any lethal effects of heat inactivated serum, normal serum or body mucus on the viability of a cultured strain of *Neoparamoeba* sp. (unpublished data).

Expression levels of pro-inflammatory cytokines and chemokines in fish have been shown to increase both locally at the site of parasitic infections and systemically in the anterior kidney and/or spleen during the course of infection. Rainbow trout infected with the parasitic ciliate *Ichthyophthirius multifiliis* had increased expression levels of the pro-inflammatory cytokines interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) and the chemokine interleukin 8 (IL-8) in the skin (site of infection) and the anterior kidney and spleen (Sigh, Lindenstrom & Buchmann 2004).

As the gill epithelium is the first host tissue to encounter *Neoparamoeba* sp. the response of gill cells to initial attachment is expected to be integral to the resulting host immune reaction. The mechanisms by which AGD causes systemic immune alterations are not known but these are probably mediated through changes in the production of various cytokines/ chemokines at the site of infection and/or the anterior kidney.

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Chapter 6 Atlantic salmon (*Salmo salar* L.) previously infected with *Neoparamoeba* sp. are not resistant to re-infection and have suppressed phagocyte function.

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Abstract

Previous studies have indicated that Atlantic salmon (*Salmo salar* L.) affected by amoebic gill disease (AGD) are resistant to re-infection. These observations were based upon a comparison of gross gill lesion abundance between previously infected and naïve control fish. Anecdotal evidence from Atlantic salmon farms in Southern Tasmania suggests that previous infection does not protect against AGD. Experiments were conducted to determine if previous infection of Atlantic salmon with *Neoparamoeba* sp. would provide protection against challenge and the immunological basis of any protection. Atlantic salmon were infected with *Neoparamoeba* sp. for 12 days then treated with a 4 hour freshwater bath. Fish were separated into two groups and maintained in either seawater or freshwater for 6 weeks. Fish were then transferred to 1 tank with a naïve control group and challenged with *Neoparamoeba* sp. Fish kept in seawater had lower mortality rates compared to first time exposed and freshwater maintained group however these data are believed to be biased by on-going mortalities during the seawater maintenance phase. Phagocyte function decreased over exposure time and freshwater maintained fish demonstrated an increased ability to mount a specific immune response. These results suggest that under the challenge conditions herein described antigen exposure via infection does not induce protection to subsequent AGD.

Keywords

Amoebic gill disease, *Neoparamoeba* sp., phagocyte function, resistance, *Salmo salar*, leucocyte proliferation assay.

Introduction

Amoebic gill disease (AGD) is associated with the protozoan parasite *Neoparamoeba* pemaquidensis, and can cause significant mortalities in sea-cage reared Atlantic salmon (*Salmo salar* L.) in South Eastern Tasmania, Australia (Munday, Zilberg & Findlay 2001). The same parasite has been implicated with AGD in other species of fish throughout the world, including Ireland, Spain, and the USA (Dyková & Novoa 2001) and has been most recently reviewed by Munday *et al* (2001).

Our understanding of the host-pathogen interactions between Atlantic salmon and *Neoparamoeba* sp. is limited. Anti-*Neoparamoeba* sp. antibodies have been detected in the serum of salmon exposed to *Neoparamoeba* sp. by various administration routes and natural exposure (Findlay, Helders, Munday & Gurney 1995; Akhlaghi, Munday, Rough & Whittington 1996; Findlay & Munday 1998; Zilberg & Munday 2001b; Gross, Carson & Nowak 2004). These antibodies indicate some method of amoebae-B-cell interaction, but the antibodies do not confer protection from disease (Akhlaghi *et al.* 1996; Findlay & Munday 1998). While systemic antibody mediated protection does not appear to occur, it has been suggested that resistance is acquired following the freshwater bathing of AGD affected fish. Protection in this instance was speculated to be due to stimulation of the innate immune system (Findlay *et al.* 1995; Akhlaghi *et al.* 1996; Findlay & Munday 1998; Zilberg & Munday 2001a; Zilberg & Munday 2001b).

Freshwater bathing has been used as a method to prophylactically control AGD of salmonids in Tasmania for a number of years (Munday *et al.* 2001). To date there

has been no report of resistance of salmon to AGD as a result of prolonged antigen exposure (infection) in the field. Atlantic salmon exposed to *Neoparamoeba* sp. by infection and kept in freshwater were not resistant to AGD when transferred back to seawater culture (D. Mitchell, unpublished). There have been no reports from Atlantic salmon farms of temporal changes in freshwater bathing intervals following infection and treatment. In contrast, evidence of resistance to AGD in the laboratory has been described following antigen exposure and use of the immunomodulator levamisole (Findlay *et al.* 1995; Findlay & Munday 1998; Findlay, Zilberg & Munday 2000).

The objectives of the present study were to determine (1) the susceptibility of Atlantic salmon to *Neoparamoeba* sp. challenge following infection, (2) the immune basis of any resistance provided by pre-exposure treatments, and (3) the effect of AGD re-infection on the immune system of fish previously exposed to *Neoparamoeba* sp.

Materials and Methods

Fish

Atlantic salmon (*Salmo salar* L.); (weight = 108.6 g + 3.5) smolt were acclimated to seawater (35 ‰, 5 μm filtered) over a period of 7 d and held in 4000 L Rathburn tanks with recirculated water and an individual biofilter system. Temperature during non-infection periods was maintained at 16 + 0.5 °C by a temperature control unit. Fish were fed daily to satiation on a commercial food pellet (Atlantic salmon grower LE, Skretting, Hobart, Australia).

Experimental Induction of AGD and Assessment of Resistance

The experimental design of this study was quite complicated and is best explained with the aid of a schematic (Figure 6-1). Following acclimation, 40 fish from the stock of 130 were transferred to an identical recirculation system and maintained until required. The remaining 90 fish were infected with *Neoparamoeba* sp. using 3300 cells L^{-1}, according to the method described by Zilberg, Gross & Munday, (2001). The tank temperature was maintained at 17.0 + 0.5 °C and salinity at 36 + 1 ‰. Fish were maintained as stated until gross signs of AGD on the gills were identified at 10 days post first exposure (DPFE). The presence of *Neoparamoeba* sp. on the gills was confirmed using immunocytochemistry on gill smears. In brief, gill smears were taken from the left gill arch, air-dried and incubated with a polyclonal rabbit anti-PA027 antibody (Howard & Carson 1994) for 30 min at 37 °C. An ABC Vectastain Kit (Vector Laboratories Inc, Burlingame, USA) was used for detection as per the manufacturer's instructions. Smears were developed with DAB (3,3'-diamino-benzidine) substrate and peroxidase buffer (Roche Diagnostics Corporation, Indianapolis, USA).

The fish in the infection tank were removed 12 DPFE and placed in a 400 L bin filled with freshwater (0 ‰) for 4 h. Oxygen was maintained between 95-100 % saturation, temperature was 16 + 1.0 °C and the stocking density was 4.5 kg m^3. Following bathing the fish were randomly separated into 2 equal groups; one group was returned to a tank of 5 µm filtered 35 ‰ seawater and the other to a freshwater (0 ‰) tank. Five weeks after the freshwater bath fish in all 3 groups were removed from their tanks, tattooed with alcian blue (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) using a Pan Jet marking gun (Wright Dental Group,

Dundee, Scotland) and returned to their respective tank treatments. Six weeks after the freshwater bath, 30 fish from the seawater maintained and naïve groups, and 36 from the freshwater maintained group were placed in a tank and infected with 3300 cells L^{-1} of *Neoparamoeba* sp. using the method described. The fish exposed to *Neoparamoeba* sp. for the first time in the challenge infection were used as susceptibility controls for the challenge experiment and comparison of survivorship. The challenge trial progressed until 1 fish remained in each of the first time exposed and freshwater maintained groups. The trial was terminated 26 days post challenge exposure (DPE).

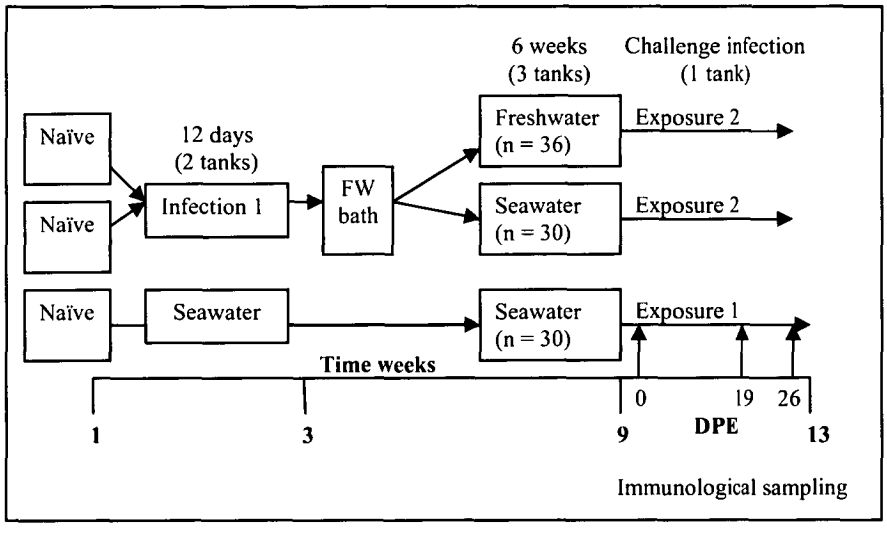

Figure 6-1 Infection and challenge design. DPE = days post *Neoparamoeba* sp. exposure in challenge infection. FW = freshwater. Immunological sample sizes; n = 5 fish per group 0 and 26 DPE, n = 3 fish 19 DPE.

Gill histology was used to identify AGD associated mortality. Gills were excised from all mortalities and moribund fish, fixed for 1 h in seawater Davidson's fixative then transferred to 70 % ethanol. The second left gill arch was used for all histological studies. Routine histological processing and sectioning techniques were used and sections stained with haematoxylin and eosin (H & E) or periodic

acid-Schiff/Alcian blue (PAS/AB). All histological sections were assessed for AGD and fish with AGD lesions associated with amoebae were considered AGD positive. If the histological section was AGD negative, a new section was cut and the gill re-assessed. The distribution of AGD associated lesions was determined and the relative numbers compared between the 8 surviving fish and 8 fish from each of the three treatment groups. Samples were chosen randomly by designating each fish with a number and then using a random number table to select the fish to be used in the quantitative histological analysis.

Sampling Procedures and Isolation of Cells

Before fish were transferred for the second infection, 5 naïve fish and 5 fish from the freshwater maintained group were terminally sampled for immunological analysis. Due to on-going mortalities in the group of fish kept in seawater after the first infection, no fish were sampled so as to maintain a minimum of 30 fish in each treatment group for the challenge infection.

During the challenge infection (19 DPE), three fish were taken from each of the seawater and freshwater maintained groups (only 1 fish surviving from the primary exposed group) for immunological studies (gills also taken) (Figure 6-1). The challenge was terminated 26 DPE and of the surviving fish from the seawater maintained group 5/6 were used for immunological studies and 6/6 for histological comparison. One fish from both the freshwater and first time exposed groups survived and were used for histological samples. All fish that died in the system or were removed moribund were assessed for clinical signs of AGD (data not shown).

Mean survival time (MST) was calculated as the day post challenge exposure (DPE) that 50 % mortalities occurred in each treatment group. Relative percent survival (RPS) was calculated as described by Amend (1981). The RPS is calculated as a ratio of percentage of mortalities in the test (vaccinate) groups to the percentage mortalities in the control group [1-(% mortalities vaccinate / % mortalities control)*100] (Amend 1981). The vaccinated groups were considered the freshwater and seawater maintained groups and the control was the first time exposed group.

Fish were euthanised with Aqui-S (Aqui-S NZ Ltd, Lower Hutt, New Zealand) and blood samples were obtained using a heparinised needle and syringe via the caudal vein. The gills were placed in seawater Davidson's fixative and the anterior kidney was removed and placed in L-15 media (Invitrogen Australia Pty Ltd, Mulgrave, Australia) containing 2 % fetal calf serum (FCS) (Invitrogen Australia Pty Ltd, Mulgrave, Australia), 0.1 % heparin (10 000 U mL^{-1}) (Sigma-Aldrich Pty Ltd, Castle Hill, Australia), 1 % penicillin/streptomycin (10 000 U mL^{-1}) (Sigma-Aldrich Pty Ltd, Castle Hill, Australia), and kept on ice until required (meshing media). Standard phagocyte isolation techniques were used as described by Secombes (1990). Phagocytes were adjusted to 1×10^7 cells mL^{-1} and 100 µL of this suspension was incubated in either 96-well flat-bottom tissue culture plates (Imbros Pty Ltd, Moonah, Australia) (respiratory burst) or chamber slides (Medos Company, Waverly, Australia) (phagocytosis) for 3 h at 18 °C.

Immunological Studies

The generation of O_2^- by anterior kidney phagocytes was determined by the reduction of nitroblue tetrazolium (NBT) (Sigma-Aldrich Pty Ltd, Castle Hill,

Australia) according to Secombes (1990). Stimulation indices (SI) were calculated as follows; SI = optical density PMA stimulated / optical density resting cells. The phagocytosis method was modified from that described by Polonio, Wolke, MacLean & Sperry (2000) and Thompson, Lilley, Chen, Adams & Richards (1999). The phagocyte monolayer and yeast cells (100 µL of 1×10^7 congo red stained yeast cells) (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) were incubated for 60 min at 18 °C. Non-phagocytosed yeast cells were removed by gentle washing with PBS and the phagocytes were fixed with 80 % methanol and stained with Quick-Dip (Histo Labs, Riverstone, Australia). Slides were examined by light microscopy at 400 × magnification. Phagocytic rate was evaluated by estimating the mean percentage of phagocytes containing at least one yeast cell, in a random count of 100 phagocytes performed in duplicate. The phagocytic index was determined by estimating the mean number of phagocytosed yeast cells, performed in duplicate.

The leucocyte proliferation assay was adapted from that described by Ardelli & Woo (2002). After plasma removal, whole blood was resuspended 1:2 in PBS (Invitrogen Australia Pty Ltd, Mulgrave, Australia) and centrifuged at 100 x ***g*** for 10 min. The buffy coat was removed using a sterile Pasteur pipette, minimising red blood cell (RBC) contamination and resuspended in PBS to 15 ml. This suspension was layered onto 5 ml Histopaque-1077 (density 1.077 + 0.001 g mL^{-1}) (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) and centrifuged at 15 °C for 30 min (400 × ***g***, no brake). Peripheral blood leucocytes (PBLs) were removed from the interface using a sterile Pasteur pipette. Cells were washed 2 × in PBS (400 × ***g***, 10 min, 4 °C) and 1 × in modified phenol red-free L-15 (Invitrogen Australia

Pty Ltd, Mulgrave, Australia) (1 % penicillin and streptomycin, P/S). Viable leucocytes were enumerated using trypan blue exclusion stain (Sigma-Aldrich Pty Ltd, Castle Hill, Australia). PBLs (2.5×10^5 cells $well^{-1}$) were incubated in 96-well, round bottom, tissue culture plates (Imbros Pty Ltd, Moonah, Australia) with L-15 (total volume of 100 µL $well^{-1}$) supplemented with 10 % L-glutamine (Sigma-Aldrich Pty Ltd, Castle Hill, Australia), P/S and 10 % fetal calf serum (FCS) and either 500, 250, 125, 62.5, 31.3, 0 µg mL^{-1} of Concavalin A (Con A) (Sigma-Aldrich Pty Ltd, Castle Hill, Australia) or *Escherichia coli* lipopolysaccharide (LPS) (Sigma-Aldrich Pty Ltd, Castle Hill, Australia). Each treatment was performed in triplicate and the plates were incubated for 4 d at 18 °C, as determined in preliminary experiments (data not shown). After incubation, proliferation was measured as described by Ardelli and Woo (2002).

Statistical Analyses

Statistical analyses were performed using SPSS version 10. Results are expressed as mean + standard error, and treatment means were considered statistically significant at the $P < 0.05$ level. Levene's test of equality of error variances was performed on all data and data failing this analysis were transformed (log) to create homogeneity of variance. One-way ANOVA was used to analyse gill histology results and the student's t-test (with limited degrees of freedom) to analyse immunological assays within groups over time or between the 0 DPE data. The Kaplan-Meier survival statistic was used to analyse the AGD challenge data.

Results

Assessment of Infection Protocol and Resistance to AGD

Fish exposed to *Neoparamoeba* sp. during the first infection were assessed for the presence of the parasite on the gills by immunocytochemistry on gill smears. This was performed on 2 occasions; 4 DPFE (n = 5) and 10 DPFE (n = 10). Smears were positive for *Neoparamoeba* sp. only 10 DPFE (10/10). There were a number (7) of mortalities during this phase of infection and histological examination confirmed that these fish were affected by AGD.

During the 6 week recovery period there were no mortalities in the group of fish maintained in freshwater following the first infection. However, 12 fish died from the group maintained in seawater after the first infection and freshwater bath. Histological examination of these fish found 11/12 were AGD positive suggesting death was a result of continuing AGD (data not shown). Histological studies found that of the mortalities from the freshwater and seawater maintained groups and first time exposed group 9, 0 and 0 % respectively, were not associated with AGD. These fish were excluded from subsequent analysis. There was a significant ($P < 0.01$) difference in the survival of fish that had been previously affected with AGD freshwater bathed and maintained in seawater for 6 weeks prior to challenge, compared to the control group (first time exposure). No difference ($P > 0.05$) was found between those fish maintained in freshwater following infection and then challenged with *Neoparamoeba* sp., and the control group (Figure 6-2). The distribution of gill filaments with AGD associated lesions from the surviving fish and the three treatment groups were significantly

different (Table 6-1). AGD lesions were detected in a high proportion of gill filaments across all treatments, first time exposed group (91 %), freshwater maintained (66 %), seawater maintained group (83 %) and surviving fish (75 %).

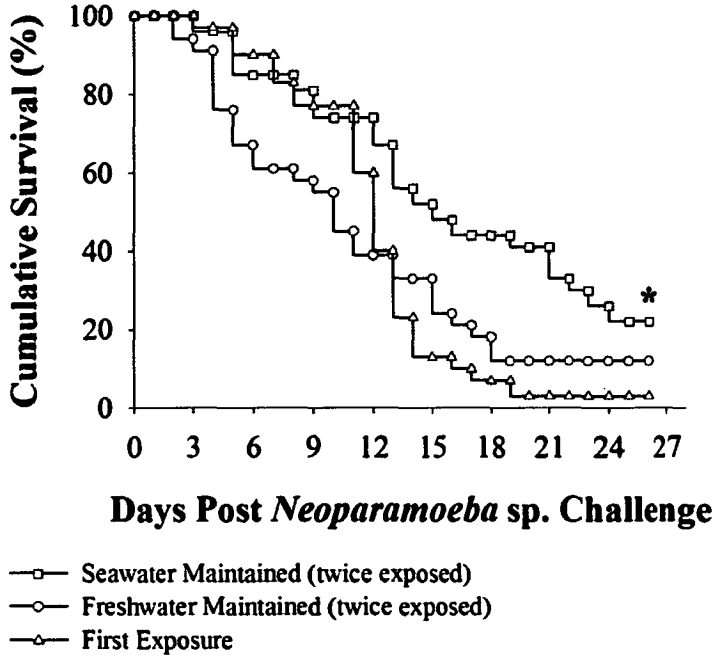

Figure 6-2 Survival following challenge with *Neoparamoeba* sp. in Atlantic salmon previously affected by AGD. Fish were once exposed, freshwater bathed and then maintained long-term (6 weeks) in seawater (n = 30) or freshwater (n = 36) prior to second time exposure and challenge. First exposure (n = 30) group were exposed to *Neoparamoeba* sp. for the first time during challenge infection and used as susceptibility control. (DPE) days post *Neoparamoeba* sp exposure during challenge infection.* statistically significant ($P < 0.01$) difference in survival compared to control group.

MST for the first time exposed, seawater and freshwater maintained groups were 12, 10 and 17 d respectively (Table 6-1). RPS values for the seawater and freshwater maintained groups were 20 and 9 respectively (Table 6-1).

Table 6-1 Histological analysis of gills, MST, RPS and non-AGD related mortalities of treatment groups after an AGD challenge infection following pre-exposure treatments.

Treatment group	Filaments affected with lesions (%)	Mean survival time (days)	Relative percent survival	Mortalities not associated with AGD (%)
Freshwater maintained	66[a]	10	9	9
Seawater maintained	83[a,b]	17	20	0
First time exposed	91[b]	12	N/A	0
Surviving fish at trial termination	75[a,b]	N/A	N/A	N/A

Different superscripts indicate results that are significantly different from each other ($P < 0.05$), n = 8 fish per treatment group for histological comparison of lesion affected filaments, N/A = not applicable to this group.

Immunological Studies

Respiratory Burst

At 0 DPE the respiratory burst activity (resting and PMA stimulated) of phagocytes from the first time exposed and freshwater maintained group were equal ($P > 0.05$) (Figure 6-3). The resting levels were statistically different ($P < 0.01$) for the freshwater maintained group between 0 and 19 DPE as were the PMA stimulated levels ($P < 0.05$) (Figure 6-3). PMA stimulated respiratory burst in the seawater maintained group were significantly different ($P < 0.01$) from 19 to 26 DPE. Similarly resting levels appeared lower from 19 to 26 DPE however they were not statistically different ($P > 0.05$) (Figure 6-3). Stimulation indices for the freshwater and seawater maintained groups at 19 DPE were 2.8 and 2.5 respectively. At 26 DPE the ratio of stimulation for the seawater maintained group was 3.0 which appeared slightly higher than the 19 DPE value ($P > 0.05$). Stimulation indices at 0 DPE for first exposed and freshwater maintained values

were 3.3 and 3.5 respectively (P > 0.05) as were the 0 and 19 DPE for the freshwater maintained group (P > 0.05).

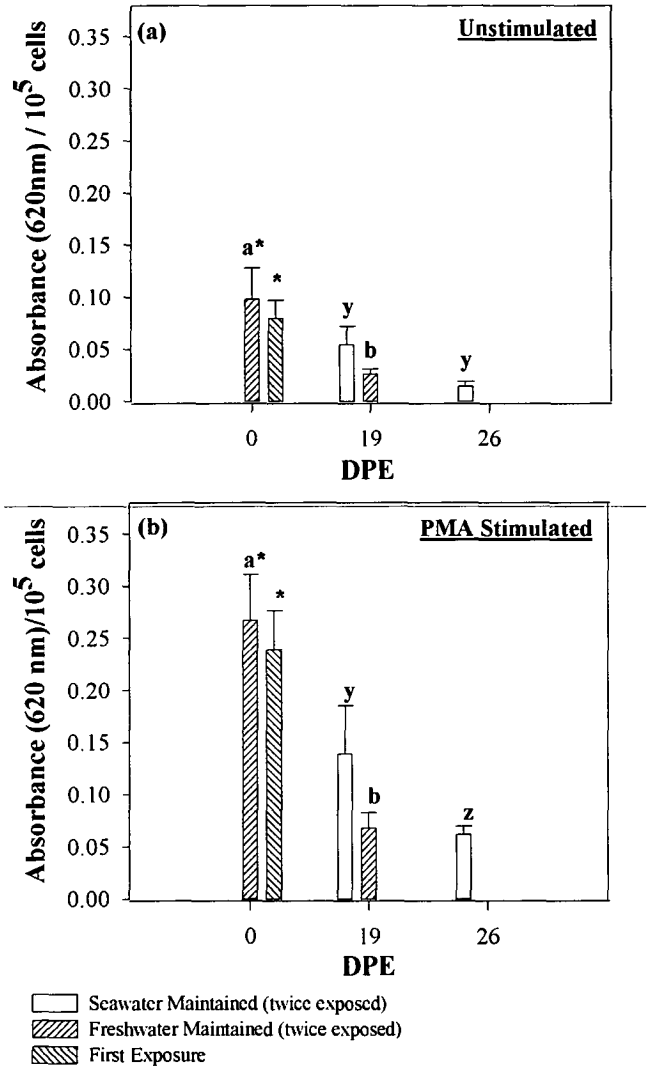

Figure 6-3 Respiratory burst response of anterior kidney phagocytes isolated from Atlantic salmon re-infected with *Neoparamoeba* sp. following treatment (freshwater bath) for an initial *Neoparamoeba* sp. infection and long-term (6 weeks) maintenance in freshwater or seawater. First exposure fish were exposed to *Neoparamoeba* sp. for the first time during challenge. Phagocytes were left at basal levels (a), or stimulated with PMA (b); (DPE) days post *Neoparamoeba* sp. exposure during challenge. Different symbol or letters indicates significant difference (P < 0.05) within group (a,b = freshwater, y,z = seawater) over time or between first time exposed and freshwater maintained group (*) on day 0 (students t-test). Range represents standard error. n = 5 fish at 0 and 26 DPE per group and n = 3 fish 19 DPE per group.

Phagocytosis

There was no significant ($P > 0.05$) change in the phagocytic rate or index of the first time exposed and freshwater maintained groups at 0 DPE (Figure 6-4 a,b). There was a significant difference ($P < 0.05$) in the phagocytic index of the seawater maintained fish between 19 and 26 DPE but there was no change in the phagocytic rate on the same days ($P > 0.05$). There was a significant difference in phagocytic rate of phagocytes from the freshwater maintained fish 0 and 19 DPE ($P < 0.01$) but no difference in phagocytic index for the same day ($P > 0.05$) (Figure 6-4 a,b).

Peripheral Blood Leucocyte Proliferation

The PBLs from each of the two groups 0 DPE responded equally to all the Con A and LPS concentrations ($P > 0.05$); (data not shown), therefore 500 $\mu g\ mL^{-1}$ was used for analysis. There was a significant ($P < 0.05$) difference in the LPS induced proliferation of PBLs from the freshwater maintained group between 0 and 19 DPE (Figure 6-5 a-c). Resting PBLs from the seawater maintained fish remained constant from 19 to 26 DPE ($P > 0.05$). LPS and Con A stimulated levels for this group remained relatively equal ($P > 0.05$). There were no differences between any of the responses to the mitogens when comparing 0 DPE results for the first time exposed and freshwater maintained group ($P > 0.05$) (Figure 6-5 a-c).

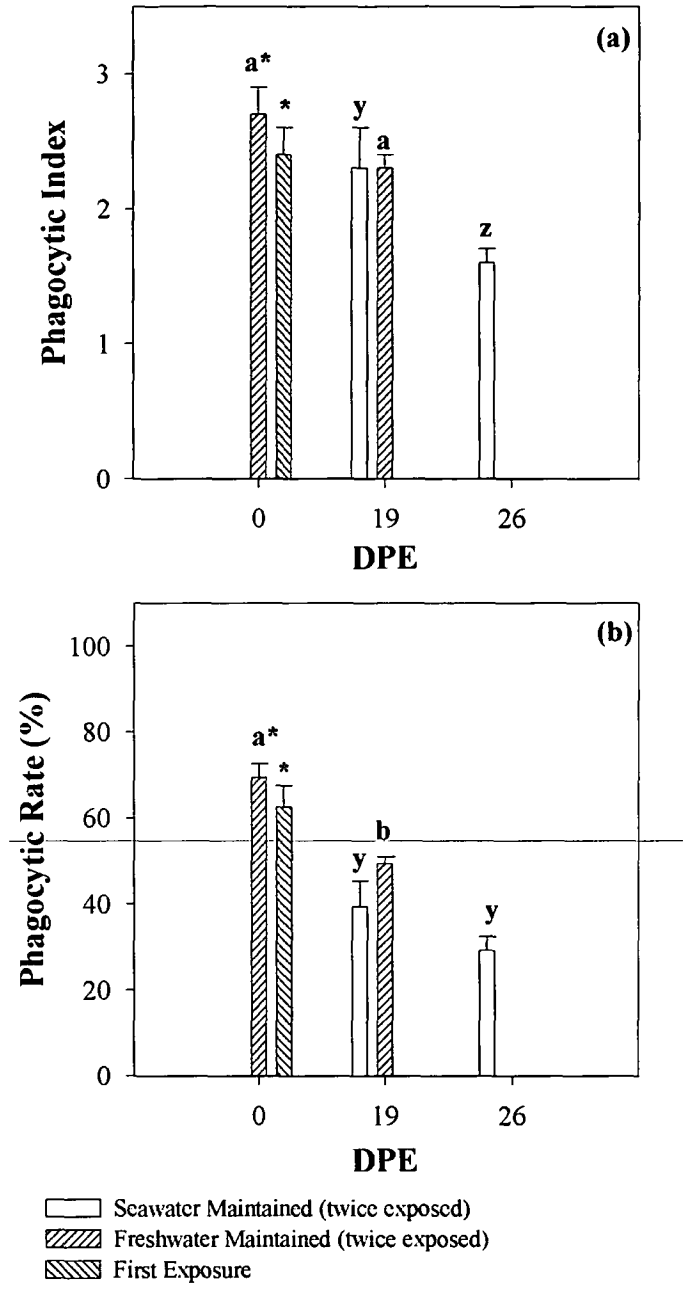

Figure 6-4 (a) Phagocytic index and (b) phagocytic rate of anterior kidney phagocytes isolated from Atlantic salmon re-infected with *Neoparamoeba* sp. following treatment (freshwater bath) for an initial *Neoparamoeba* sp. infection and long-term (6 weeks) maintenance in freshwater or seawater. First exposure fish were exposed to *Neoparamoeba* sp. for the first time during challenge. (DPE) days post *Neoparamoeba* sp. exposure. Different symbol or letters indicates significant difference ($P < 0.05$) within group (a,b = freshwater, y,z = seawater) over time or between first time exposed and freshwater maintained group (*) on day 0 (students t-test). Range represents standard error. n = 5 fish on 0 and 26 DPE per group and n = 3 fish 19 DPE per group.

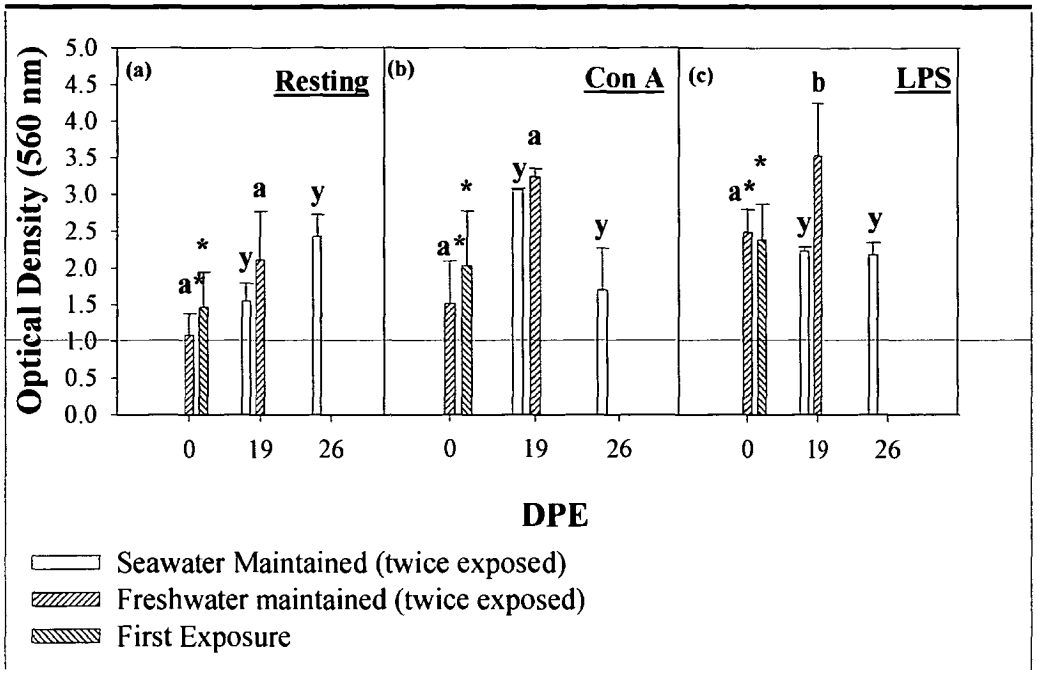

Figure 6-5 Mitogenic response of peripheral blood leucocytes isolated from Atlantic salmon re-infected with *Neoparamoeba* sp. following treatment (freshwater bath) for an initial *Neoparamoeba* sp. infection. Fish were maintained in either seawater or freshwater for 6 weeks until the second exposure; (a) resting (non-stimulated cells) cells, (b) Con A stimulated cells, and (c) LPS stimulated cells; (DPE) days post second *Neoparamoeba* sp. exposure for challenge infection. Range represents standard error. Different symbols and letters indicate significant differences ($P < 0.05$) within groups (a,b = freshwater, y,z = seawater) over time and between first time exposed and freshwater maintained group (*) on day 0 (students t-test).

Discussion

Previous studies have reported resistance of Atlantic salmon to AGD following freshwater maintenance (Findlay *et al.* 1995; Findlay & Munday 1998). However, no resistance to AGD has been reported in the field as a result of freshwater treatments. This study was designed to use infection as a method to determine if practical resistance (protection from death as a result of AGD) could be induced in Atlantic salmon from previous infection and to characterise the resistance if present on an immunological level. In contrast to the previous reports, fish in this study were not protected upon antigen re-exposure and the results were more consistent with on-farm observations.

Fish maintained in seawater for 6 weeks following first time exposure and freshwater bathing to treat AGD had significantly higher survivorship when challenged with re-infection compared to first time exposed fish. Conversely, fish treated with a freshwater bath and then long-term freshwater maintenance before challenge did not have significantly different survivorship compared to the first time exposed group. This is in contrast to previous results where fish maintained in freshwater were less susceptible to re-infection with AGD (Findlay *et al.* 1995; Findlay & Munday 1998). In the previous studies there were no reports of mortalities in the seawater maintained group. In the present study 11 AGD positive fish died during the seawater maintenance phase. It is believed that these mortalities significantly contributed to the lower mortality rate in this group of fish. It is likely that the remaining fish had a reduced susceptibility to AGD related death not as a result of increased resistance to disease but because the

most susceptible fish in this group died prior to challenge. Consequently, the less susceptible fish in this treatment group were left for the challenge part of the trial.

It is recommended that the relative percent survival of a treatment group be over 60 (statistically significant at P = 0.01) before the specific treatment (or vaccine) will result in protection in the field (Amend 1981). The RPS of the seawater maintained group was 20, indicating that although there was a statistical significance in survivorship these fish were not practically protected from disease. The RPS of the freshwater maintained group was very low (9) and there was obviously no practical resistance to AGD in this group despite the lower percent cumulative mortality relative to the control group. As there were few mortalities in the previous studies (Findlay *et al.* 1995; Findlay & Munday 1998) it may not have been practical to assess resistance, as control mortality should have been in excess of 60 % (Amend 1981; Nordmo 1997). It has been suggested that the "resistance" of previously exposed AGD affected fish may be negated by excessive challenge (Munday *et al.* 2001). For example when previously exposed fish are infected with a low intensity *Neoparamoeba* sp. infection "resistance" may be demonstrated. However when this infection is of a high intensity with significant mortalities, as may be seen in the field or in laboratory studies that use an infectious mucus isolate to induce AGD, previously exposed fish are as equally susceptible as first time exposed fish. The difference between the freshwater maintained and first time exposed group mortalities was modest and it is believed that this difference could be manipulated by altering the challenge model and other variables. Water temperature and salinity have been shown to play a significant role in AGD outbreaks (Clark & Nowak 1999). Temperature in this

current trial was maintained at 17 + 0.5 °C during infection, compared to 14 °C (Findlay *et al.* 1995; Findlay & Munday 1998). In the previous trial, the infection was performed in seawater, however the exact salinity is not given and as the seawater in the Aquatic Centre (University of Tasmania) where all experiments were done can vary from 28 – 35 ‰ (K. Gross unpublished observations), a lower salinity may have been used in the previous trial. When combined with the lower temperature, infection may have been retarded. The experiment described here and previous studies used different challenge models. Fish were infected via cohabitation in the previous studies and not by direct exposure to an infectious gill mucus isolate, a method that induces infection much quicker than co-habitation and is much more controlled (Zilberg *et al.* 2001). This is reflected in the mean survival data. The first time exposed group in this study had a mean survival time of 12 days whereas in the previous studies first time exposed fish were still alive 3 and 4 weeks after exposure (Findlay *et al.* 1995; Findlay & Munday 1998).

There was no significant difference in the proportion of filaments affected by AGD lesions in fish that were still alive at the termination of the trial compared to any of the other 3 groups. Whilst these fish were less susceptible they were not resistant to disease (presenting as gill lesions). The fish that had been maintained in freshwater prior to re-infection had statistically lower percentage of AGD affected filaments compared to fish from the first time exposed group. This in part replicates previous findings (Findlay *et al.* 1995; Findlay & Munday 1998). It is believed a stress effect due to salinity change at the beginning of the challenge may have played a role in their initial susceptibility.

The immune parameters assessed at 0 DPE for the freshwater maintained group of fish and the first time exposed group were not significantly different ($P > 0.05$), indicating that maintenance in freshwater after an initial infection does not enhance innate immunity as has been suggested (Findlay *et al.* 1995; Findlay & Munday 1998). Freshwater maintained fish had an increase in resting, B- and T-cell proliferative abilities following second time exposure, suggesting that in this population of fish *Neoparamoeba* sp. infection altered the mitogenic responsiveness of PBLs. Macrophages and epithelial cells are responsible for producing interleukin-1 which has a direct effect on T and B-cell activation (Weir & Stewart 1997). Recent studies have identified up-regulation of systemic (hepatic) interleukin-1β (IL-1β) mRNA in AGD affected fish (A. Bridle, unpublished data). An increase in IL-1β production may influence proliferation in resting cells as well as the mitogenic responsiveness of PBLs as observed here. Whilst freshwater maintained fish showed an increased ability to mount an acquired immune response after re-exposure, they were not protected from AGD. In contrast, PBLs from fish in the seawater maintained group did not have an enhanced ability to respond to mitogens. T-cell proliferative abilities decreased over exposure time, but the proliferative abilities of B-cells was not altered. This suggests that chronic AGD may suppress specific-cell mediated response, possibly since these fish did not have a convalescence period following the initial infection. A long-term suppression of specific-cellular immune responses as a result of chronic infection may render these fish more susceptible to opportunistic pathogens.

There appeared to be suppression of the respiratory burst responses of phagocytes from the seawater and freshwater maintained groups over the course of the challenge, however AGD appears to affect the resting level, not the ability of phagocytes to respond to protein kinase C mediated activation (PMA). Given that the stimulatory ability of phagocytes may be directly proportional to the resting intracellular superoxide concentration, suppression may have downstream effects, compromising an innate immune response. As the immune response of the seawater maintained groups declined over time, any protection within this group was not provided by the anterior kidney phagocyte population. Phagocytes from the anterior kidney may be actively recruited to the site of infection and/or into the peripheral blood, leaving immature or resting cells in the haematopoietic tissue resulting in apparent "suppression" of phagocyte function. Alternatively a primary and/or secondary stress response during AGD may influence pronephros phagocyte function. For example, Atlantic salmon infected with sea lice (*Lepeophtheirus salmonis*) exhibited a significant decrease in respiratory burst function and phagocytosis at 21 days post exposure, coinciding with an elevation in plasma cortisol and glucose (Mustafa, MacWilliams, Fernandez, Matchett, Conboy & Burka 2000). It was surmised that the greatest effect of sea lice on Atlantic salmon was during the latter stages of their life-cycle, at which point stress mediates immune suppression (Mustafa *et al.* 2000).

Decreases in phagocyte function are not uncommon during the course of infection in fish (Angelidis, Baudin-Laurencin, Quentel & Youinou 1987). In addition, changes in peripheral blood and anterior kidney leucocyte populations during infection are well documented. Most recently, infection of carp (*Cyprinus carpio*

L.) with the haemoflagellate *Trypanoplasma borreli* resulted in an increased proportion of neutrophilic granulocytes and lymphoblasts in the peripheral blood (Scharsack, Steinhagen, Kleczka, Schmidt, Korting, Michael, Leibold & Schuberth 2003). The proportion of lymphoblasts in the anterior kidney increased, however the relative number of neutrophilic granulocytes decreased. A reduction in cellular innate immune response (oxidative burst and phagocytosis) and an increase in proliferation of rainbow trout (*Oncorhynchus mykiss*, Walbaum) leucocytes during proliferative kidney disease has also been reported (Chilmonczyk, Monge & de Kinkelin 2002). The depression of the granulocyte function accounted for the decrease in innate immunity and helped to explain the poor prognosis. It is hypothesised that a similar response may occur in Atlantic salmon during AGD. The stage at which phagocyte function decreases is considered the point at which the host becomes physiologically compromised as concurrent mortalities occur (K. Gross, unpublished data).

Together, these data described here support on-farm observations that fish bathed and allowed to recover from AGD in freshwater are not resistant to re-infection. Under the challenge conditions described there is no resistance provided by prior infection with *Neoparamoeba* sp. regardless of whether the fish are allowed to fully convalesce or if they remain actively infected. AGD appears to alter anterior kidney phagocyte function although it is not known if this is due to migration of phagocytes away from the kidney, inflammatory cytokines, stress or another unidentified effect of infection on phagocyte function.

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Chapter 7 The effect of plasma and mucus from AGD-affected and unaffected Atlantic salmon (*Salmo salar* L.) on the *in vitro* viability of *Neoparamoeba* spp.

Manuscript in preparation

Abstract

There have been many reports of the lethal effects of fish serum/plasma and/or mucus on ectoparasites. Here we investigated whether plasma or mucus from Atlantic salmon (*Salmo salar*) affected the viability of *Neoparamoeba* spp.. *Neoparamoeba* spp. were isolated from the gills of amoebic gill disease (AGD)-affected Atlantic salmon and incubated with plasma (heat-inactivated or untreated) or mucus from AGD-affected or unaffected Atlantic salmon for 1 and 4 hours. There was a significant decline in the viability of *Neoparamoeba* spp. after incubation with plasma from either AGD-affected and unaffected Atlantic salmon at both 1 and 4 h post-incubation ($P < 0.05$). This may be mediated by the plasma osmolality as the *Neoparamoeba* spp. were also non-viable in L15. There was evidence that agglutinins were present in the plasma from AGD-affected fish as when *Neoparamoeba* spp. were incubated with neat, 1:5 and 1:10 L15-diluted plasma they agglutinated. Mucus from infected and uninfected fish had no effect on the viability of *Neoparamoeba* spp. ($P > 0.05$). These results suggest that the osmolality of plasma is a significant impediment when investigating the effect of incubating *Neoparamoeba* spp. with plasma. The osmolality causes a decline in amoebae viability and this may mask the effect of any factors that are present in the plasma and which can kill the amoebae. Under the experimental conditions herein described there are no factors present in plasma cutaneous mucus that are capable of affecting the viability of *Neoparamoeba* spp.. Together, these results indicate that future research could focus on identifying techniques to investigate the interactions between *Neoparamoeba* spp. and host factors in an environment that will suit the physiology of both.

Introduction

The innate immune system of fish is comprised of cellular and humoral components (reviewed by Magnadottir 2006). Humoral constituents include serum anti-proteases, lytic enzymes, complement and mucosal and serum agglutinins such as C-type lectins and pentraxins (reviewed by Magnadottir 2006). Sera and mucus from immune and non-immune individuals has been documented to be an effective killer of parasites in numerous teleost disease models (Smith, Noga, Levy & Gerig 1993; Buchmann 1998; Harris, Soleng & Bakke 1998; Mehta & Woo 2002; Rubio-Godoy, Porter & Tinsley 2004).

Serum complement is capable of killing parasites such as gyrodactylids through activation of the alternative or lectin mediated pathways (Buchmann 1998; Harris *et al.* 1998). The kinetoplastid *Cryptobia salmositica* stimulates the acquired immune response to produce specific antibodies which bind to the parasite and in-turn activate complement via the classical pathway (Mehta & Woo 2002). While the monogenean *Discocotyle sagittate* stimulates complement through activation of the alternative pathway (Rubio-Godoy *et al.* 2004). Serum from immunised tilapia (*Oreochromis aureus*) caused agglutination of live *Amyloodinium ocellatum* however, heat-inactivation of the serum abated this effect but did not completely eliminate it which indicates complement was not the only factor affecting the parasite (Smith *et al.* 1993).

The cutaneous mucosal immune system is an important component of the fish immune system as it is the first line of defence against ectoparasites. For example; mucus from the skin of Atlantic salmon (*Salmo salar*) affects the

viability of the monogenean *Gyrodactylus salaris* (Harris *et al.* 1998). Further studies on the significance of mucus in eliminating parasites has reported a negative correlation between the density of mucous cells and the number of monogeneans on the surface of rainbow trout (*Oncorhynchus mykiss*) (Buchmann & Bresciani 1998). Channel catfish (*Ictalurus punctatus*) that survive infection with *Ichthyophthirius multifiliis* may be protected from subsequent infections and this protection has been correlated to the presence of humoral antibodies in the cutaneous mucus (Maki & Dickerson 2003).

Amoebic gill disease (AGD) is associated with the protozoan ectoparasite *Neoparamoeba* spp. (Munday, Zilberg & Findlay 2001; Dyková, Nowak, Crosbie, Fiala, Pecková, Adams, Macháèková & Dvoráková 2005). There have been few published studies regarding the *in vitro* interactions of *Neoparamoeba* spp. and host immune factors. The published literature investigates the effects of *Neoparamoeba* spp. on host cells (Butler & Nowak 2004; Gross, Alcorn, Murray, Morrison & Nowak 2006; Lee, Van Es, Walsh, Rainnie, Donay, Summerfield & Cawthorn 2006) as opposed to the effect of the host on the parasite. AGD is typically associated with an environmental salinity of 35 ‰ (Munday, Foster, Roubal & Lester 1990) and salinity has been identified as the key environmental factor affecting the prevalence of AGD (Clark & Nowak 1999). Furthermore environmental salinity is imperative for the survival of *Neoparamoeba* sp (Douglas-Helders, Nowak & Butler 2005) *in vitro*. Due to the precedence of plasma and mucus from salmonids being able to kill ectoparasites the effect of untreated and heat-inactivated plasma or mucus on the viability of *Neoparamoeba* spp. was investigated.

Materials and Methods

Fish

Mucus and plasma samples were collected from 10 AGD-affected (189 ± 12 g) and unaffected (186 ± 15 g) Atlantic salmon (*Salmo salar*). Unaffected (naive controls) fish had not been exposed to seawater and therefore never exposed to *Neoparamoeba* spp.. AGD-affected fish were from the same population of fish as the naive controls but were infected by co-habitation for 6 d, with AGD-affected Atlantic salmon.

Due to the difficulty associated with obtaining gill mucus, body mucus was isolated from the fish as it is not considered biochemically different from gill mucus (Shepard 1994). AGD-affected and unaffected fish were lightly anaesthetised using AQUI-S (0.01 % v v^{-1}) and the skin mucus collected from the body by gentle scraping of the fish skin as described by Roberts and Powell (2005). Mucus from each group (AGD-affected and unaffected) was pooled into aliquots and stored at -80 °C until use. Plasma samples were collected by caudal vein puncture using heparinised, 27-gauge needles and syringes. One aliquot of plasma was heat-treated to inactivate complement (20 min at 44 °C) (Sakai 1981) and the plasma samples were stored at -80 °C until required. Before use, plasma samples were diluted 1:2, 1:5 and 1:20 with L15. Seawater and Leibovitz's medium (L15) were used as positive and osmolality controls respectively. The osmolality of the plasma, mucus, L15 and seawater were measured using a vapour pressure osmometer (Wescor Vapro) (Table 7-1).

Isolation of gill associated *Neoparamoeba* spp.

Neoparamoeba spp. were isolated from the gills of AGD-affected Atlantic salmon using a plastic adherence method (Morrison, Crosbie & Nowak 2004). Isolated *Neoparamoeba* spp. were resuspended in sterile, filtered (0.2 μm) seawater in a tissue culture flask and incubated at 18 °C overnight. Due to the numbers of *Neoparamoeba* spp. isolated, this study was performed on two separate occasions using two isolates of *Neoparamoeba* spp.. Consequently the numbers of *Neoparamoeba* spp. used for the plasma and mucus studies were different.

When investigating *in vitro* interactions between a host and pathogen it is imperative to ensure optimal conditions for both. One major difficulty when investigating the effect of host factors on *Neoparamoeba* spp. is maintaining optimal osmolality conditions for both. *Neoparamoeba* spp. are obligate marine organisms (sea water approximate osmolality of 1000 mmol kg^{-1}) and host factors are present in physiological osmolalities of around 350 mmol kg^{-1}. *Neoparamoeba* spp. are viable at salinities down to 10 ‰ (Kent, Sawyer & Hedrick 1988), or approximately 330 mmol kg^{-1}, close to physiological osmolality. It has been demonstrated that whilst serum antibody function is not affected by osmolality within the 100-400 mmol kg^{-1} range, extremes of pH (< 7 and >8) and an osmolality greater than 500 mmol kg^{-1} results in a decrease in antibody affinity between 55-64 % (Bricknell, Bisset & Bowden 2002). Although not looking specifically at immunoglobulins in plasma or mucus it was decided that changing the osmolality of the serum would not allow optimal conditions for investigating the effect of host components on *Neoparamoeba* spp..

Table 7-1 Sample osmolality (mmol kg^{-1})

Sample	Osmolality of plasma from AGD-unaffected fish (mmol kg^{-1})	Osmolality of plasma from AGD-affected fish (mmol kg^{-1})
Neat plasma (untreated)	320	394
1:2 L15 diluted plasma (untreated)	318	348
1:5 L15diluted plasma (untreated)	345	341
1:20 L15diluted plasma (untreated)	330	342
Heat-inactivated plasma (neat)	330	380
Mucus (neat)	703	746
L15	325	330
Seawater	987	987

Effect of plasma on *Neoparamoeba* spp. viability

Based upon pilot studies (data not shown) when *Neoparamoeba* spp. are incubated with L15 the viability of the cells did not decline below 10 %. L15 was therefore chosen for use in this study because a proportion of the *Neoparamoeba* spp. would remain viable in this media and the integrity of the host factors would remain intact. The day following isolation, cells were counted and 5000 viable *Neoparamoeba* spp. were added to each well of a 96-well microtitre plate. The *Neoparamoeba* spp. were allowed to adhere (approximately 1 h) after which time non-adherent cells and seawater were removed. Adhered *Neoparamoeba* spp. were incubated with 200 µL of untreated neat plasma, heat-inactivated plasma, 1:2, 1:5 and 1:20 diluted (L15) untreated plasma, L15 only and seawater in quadruplicate wells. After 1 h *Neoparamoeba* spp. were mixed with a pipette and 50 µL removed and added to 50 µL trypan blue (0.25 % w v^{-1} seawater). The number of viable cells was then counted using a hemocytometer. This process was repeated after 4 h incubation.

Effect of mucus on *Neoparamoeba* spp. viability

Following isolation of the *Neoparamoeba* spp., 3900 cells were added to each well of a plastic 96-well microtitre plate. The *Neoparamoeba* spp. were allowed to adhere for 1 h and non-adherent cells and seawater removed. Pooled mucus from naive or infected fish was added (200 µL per well) to quadruplicate wells along with a seawater control. Cell viability was assessed as described above.

Statistical analyses

Results were analysed using one-way ANOVA at 1 and 4 h incubation time Statistical analyses were performed using SPSS version 10. Results are expressed as mean ± standard error and treatment means were considered statistically significant at the $P < 0.05$ level. Levene's test of equality of error variances was performed on all data prior to analysis and data failing this test were transformed (log) to create homogeneity of variance.

Results

All fish removed from the cohabitation infection tank had significant white patches on the gills, indicative of AGD. Histology of the gills found 83 % (± 5) of filaments were affected with AGD-lesions (data not shown).

Compared to the seawater only control there was a significant ($P < 0.05$) effect of plasma (heat-treated, untreated and diluted) from AGD-unaffected fish on *Neoparamoeba* spp. viability at 1 and 4 h post-incubation (Figure 7-1). The *Neoparamoeba* spp. viability decreased in the plasma and L15 treatments between 1 and 4 h ($P < 0.05$). There was a significant effect of osmolality on *Neoparamoeba* spp. viability as shown by the effect of L15 alone ($P < 0.05$) on

Neoparamoeba spp. viability compared to the seawater control (Figure 7-1). *Neoparamoeba* spp. viability was significantly greater when incubated with neat and 1:2 diluted plasma compared to L15 after 1 h ($P < 0.05$) however after 4 h incubation there was no difference between any of the treatments except when compared to the seawater control only ($P > 0.05$) (Figure 7-1).

Neoparamoeba spp. that were incubated in plasma isolated from AGD-affected fish (Figure 7-2) agglutinated in the neat, 1:2 and 1:5 dilutions of the untreated plasma. This was seen after the cells had been pipetted multiple times to remove them from the plastic and then removed for counting. It was only within the mentioned groups that this agglutination of the *Neoparamoeba* spp. was noted and for all replicates and treatment groups the process of pipetting was equal. No agglutination was seen in the 1:20 diluted plasma or the heat-inactivated plasma. Due to agglutination of the *Neoparamoeba* spp. viability counts were difficult to perform and no statistical analysis was done on these data. There was significant ($P < 0.05$) decrease in survival of *Neoparamoeba* spp. when incubated in 1:20 untreated plasma compared to the seawater control treatment (Figure 7-2). As there was no agglutination of the *Neoparamoeba* spp. incubated in the 1:20 untreated plasma, L15 and the seawater statistical analysis was performed on these data. After 1h incubation the L15 and heat-inactivated plasma had a significant effect on *Neoparamoeba* sp. viability ($P < 0.05$) compared to the seawater control (Figure 7-2). There was no effect of 1:20 diluted plasma on *Neoparamoeba* spp. compared to the seawater control ($P > 0.05$). Significantly more *Neoparamoeba* spp. survived in the 1:20 diluted plasma than the L15 or heat-inactivated plasma ($P < 0.05$) (Figure 7-2).

Incubation with L15 for 4h resulted in significantly less *Neoparamoeba* spp. viability ($P < 0.05$) compared to the seawater control and the 1:20 diluted plasma (Figure 7-2). Heat-inactivated plasma did not have an effect on viability compared to L15 ($P > 0.05$), but less *Neoparamoeba* spp. survived in heat-inactivated plasma than in seawater ($P < 0.05$) (Figure 7-2).

There was no significant ($P > 0.05$) effect of mucus from AGD-affected or unaffected Atlantic salmon on *Neoparamoeba* spp. viability compared to the seawater control (Figure 7-3). Furthermore, there was also no significant ($P > 0.05$) effect of incubation time on *Neoparamoeba* spp. viability (Figure 7-3).

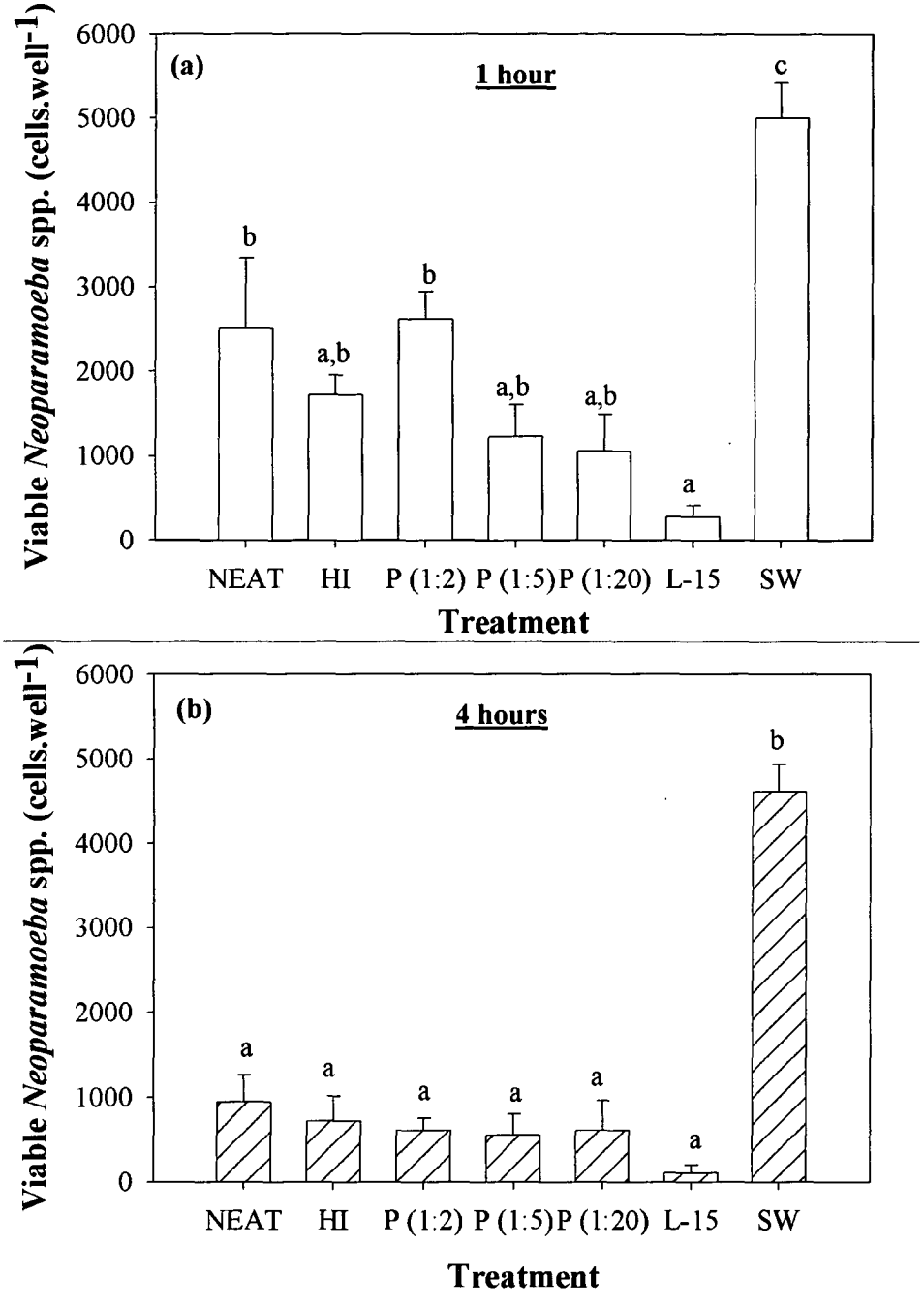

Figure 7-1 The effect of plasma from AGD-unaffected fish on *Neoparamoeba* spp. viability after 1 and 4 h incubation. Plasma was obtained from AGD-unaffected Atlantic salmon, treated and incubated with 5000 cells well^{-1} of *Neoparamoeba* spp. for either 1 or 4 hours. After incubation the number of viable cells was counted. Neat = neat untreated plasma, HI = heat-inactivated plasma, P (1:2) = untreated plasma diluted 1:2, P (1:5) = untreated plasma diluted 1:5, P (1:20) = untreated plasma diluted 1:20, L15 = osmolality control, SW = positive control (sea water treatment). Different letters indicate significant ($P < 0.05$) difference in viable *Neoparamoeba* spp. between treatments after 1 h incubation. Different letters indicate significant ($P < 0.01$) difference in viable *Neoparamoeba* spp. between treatments after 4 h incubation.

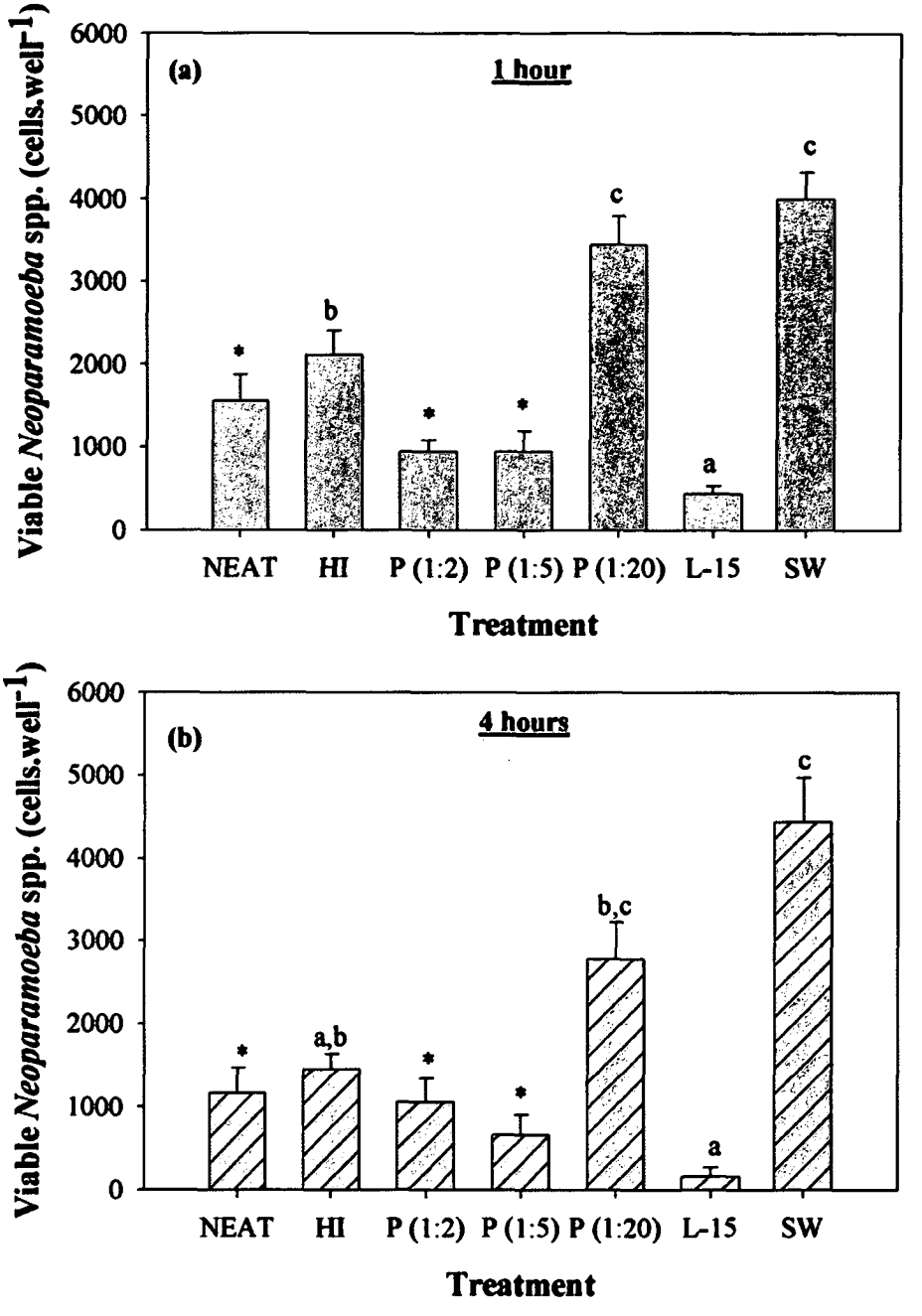

Figure 7-2 The effect of plasma from AGD-affected fish on *Neoparamoeba* spp. viability after 1 and 4 h incubation. Plasma was obtained from AGD-affected Atlantic salmon, treated and incubated with 5000 cells well^{-1} of *Neoparamoeba* spp. for either 1 or 4 hours. After incubation the number of viable cells was counted. Neat = untreated neat plasma, HI = heat-inactivated plasma, P DIL (1:2) = untreated plasma diluted 1:2, P (1:5) = untreated plasma diluted 1:5, P (1:20) = untreated plasma diluted 1:20, L15 = osmolality control, SW= positive control (sea water treatment). Different letters indicate significant ($P < 0.05$) difference in viable *Neoparamoeba* spp. between treatments after 1 h incubation. Different letters indicate significant ($P < 0.01$) difference in viable *Neoparamoeba* spp. between treatments after 4 h incubation. * Indicates treatments where *Neoparamoeba* spp. agglutinated and therefore no statistical analysis was performed.

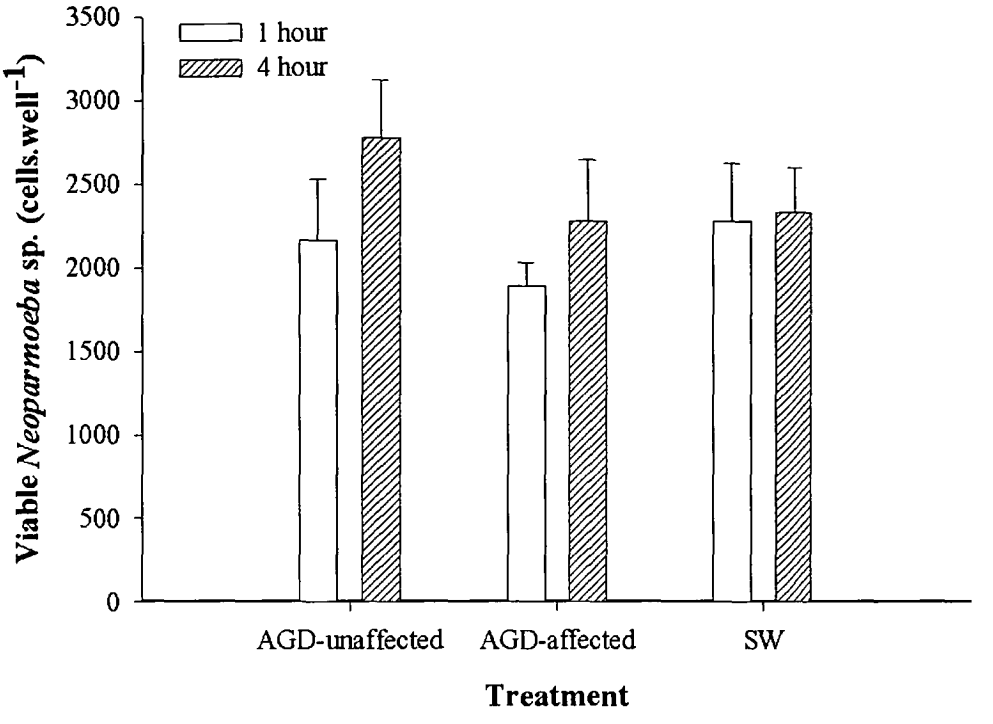

Figure 7-3 The effect of mucus isolated from AGD-unaffected and affected Atlantic salmon on *Neoparamoeba* spp. viability after 1 and 4 h incubation. Cutaneous mucus was obtained from AGD-affected and unaffected Atlantic salmon and incubated with 3900 cells well^{-1} of *Neoparamoeba* spp. for either 1 or 4 hours. After incubation the number of viable cells was counted. SW = positive control (sea water treatment). No significant ($P > 0.05$) differences between treatments or over time were identified.

Discussion

The evidence in this study indicates that the osmolality of plasma was the important factor in the reduced viability of *Neoparamoeba* spp. and not components within the plasma. If there were factors present in plasma that could kill *Neoparamoeba* spp., reduced viability in plasma treated-amoebae below that of amoebae incubated with the osmolality control (L15) would be expected. Also, as heat-inactivation of plasma did not affect *Neoparamoeba* spp. viability, suggesting that complement was not associated with the decline in viability fish complement is not functional when heated to 44°C (Sakai 1981). The increased viability of *Neoparamoeba* spp. incubated in the higher concentrations of plasma may suggest that the plasma had a buffering effect on the *Neoparamoeba* spp., protecting them from the negative effects of osmolality.

In the current study, plasma was diluted with L15 knowing that the osmolality of the suspensions would be detrimental to *Neoparamoeba* spp. as it is an obligate marine organism (Page 1987). Dilution of the plasma with sea water would have provided an environment more suited to *Neoparamoeba* spp.. However as humoral components such as immunoglobulins have a limited salinity and pH tolerance range (Bricknell *et al.* 2002) dilution of plasma with seawater (or other substance with osmolality greater than 400mOsm) could result in decreased function of the humoral factors and the aim of this study was to identify the effect of plasma on *Neoparamoeba* spp. viability. Therefore so as to maintain the integrity of all plasma components the plasma was diluted with L15 instead of sea water.

Agglutination of *Neoparamoeba* spp. incubated with plasma suggests that agglutinins are present in plasma from AGD-affected fish. Some agglutinins such as C-reactive protein (CRP) and serum amyloid protein (SAP) are lectins which are present during the acute phase response (APR) to infection (Magnadottir 2006). However, no up-regulation of hepatic APR proteins in AGD-affected Atlantic salmon has been identified as demonstrated by equal levels of serum amyloid A (SAA) and SAP-like pentraxin mRNA expression in AGD-affected and unaffected Atlantic salmon (Bridle, Morrison, Cupit Cunningham & Nowak 2006). As the proportion of gill filaments affected with AGD-associated lesions were not reported it is difficult to compare the severity of that infection to the present study, as intensity of infection may play a role in the APR (Bayne & Gerwick 2001; Simko, Falk, Poppe & Ferguson 2001; Gerwick, Steinhauer, La Patra, Sandell, Ortuno, Hajiseyedjavadi & Bayne 2002; Hoole, Lewis, Schuwerack, Chakravarthy, Shrive, Greenhough & Cartwright 2003). SAA and SAP-like pentraxin are only two members of the APR protein family others such as CRP may also be responsible for agglutinins in the plasma from AGD-affected fish. From visual observations of the agglutinated *Neoparamoeba* spp. it was not evident that the *Neoparamoeba* spp. had been lysed. This suggests that complement was not stimulated by the presence of these agglutinins.

Cutaneous mucus from Atlantic salmon can kill the monogenean parasite *Gyrodactylus salaris in vitro* (Harris *et al.* 1998). In the present study Atlantic salmon cutaneous mucus did not kill *Neoparamoeba* spp.. As gill mucus is the first protective barrier that *Neoparamoeba* spp. encounter when colonising the fish it may be expected that mucus would have no effect on parasite viability.

Monogenean and crustacean ectoparasites can modulate mucus production during attachment by reducing the number of mucous cells in the skin of the host (Wells & Cone 1990; Jones 2001). During infection with *Neoparamoeba* spp. the number of mucous cells in affected gills increases during chronic infection (Nowak & Munday 1994; Zilberg & Munday 2000; Adams & Nowak 2003; Roberts & Powell 2003) the mechanism of which is yet to be elucidated. These results suggest that there are no substances present in cutaneous mucus that prevent *Neoparamoeba* spp. from residing on the body. Specification of attachment may be due to tissue type, protection from water flow past the gill or other.

Under the conditions of this study there does not appear to be any deleterious affect of mucus or plasma on the *in vitro* viability of *Neoparamoeba* spp. The results of this investigation suggest that there are no significant humoral factors present in plasma or mucus capable of eliminating *Neoparamoeba* spp. from the gill of infected fish although 7 days may not be enough time for specific antibodies to develop in the plasma of AGD-affected fish. The role of agglutinins, specific antibodies and complement in plasma of AGD-affected fish may be areas for future research to focus. Also, development of techniques to investigate *Neoparamoeba* spp. *in vitro* despite its requirement for a marine environment would be advantageous for future *in vitro* host-pathogen interaction studies.

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Chapter 8 General Discussion

Gill cell (GC) responses during infection

Viable gill cells (GCs) isolated from AGD-affected and unaffected Atlantic salmon (*Salmo salar*) were capable of phagocytosing yeast cells but not actively producing reactive oxygen species (ROS) when stimulated or chemotactic migration. It was suggested that nitroblue tetrazolium (NBT) conversion occurred in the GC suspensions due to non-leucocytic ROS production (chapter 2). It was further surmised that the lack of significant ROS production or chemotaxis may have been because few of the cells in the GC suspension were phagocyte-like and this was supported by the dominant leucocyte population in gill cells isolated from rainbow trout (*Oncorhynchus mykiss*) in a similar study, being lymphocytes (Lin, Ellis, Davidson & Secombes 1999).

Yeast cells may have been phagocytosed by cells other than leucocytes, for example pillar cells (Chilmonczyk & Monge 1980) and epithelial cells (Moore, Ototake & Nakanishi 1998). GCs isolated from both normal and AGD-affected fish were unable to produce ROS above basal levels or chemotactically migrate, indicating that there are not more leucocytes in the gill of AGD-affected fish. Other evidence indicates that leucocyte populations do not change in the gill during AGD. The stability in Ig bearing cell numbers in the gill during AGD (chapter 3) indicates that Ig bearing cells did not proliferate at the site of infection or migrate to it. The results presented in chapters 2 & 3 suggest that a significant cellular infiltration of immune cells both phagocyte-like and Ig bearing into the

gill does not occur during AGD. Furthermore on histological examination the gills of affected fish did not have an obvious cellular infiltration.

Complete perfusion of the gills was essential to ensure that peripheral blood leucocytes did not influence the results (chapter 2). During pilot studies for the assessment of GC function it was difficult to perfuse the gills of Atlantic salmon with severe AGD as the fish died before the perfusion process was complete. This response by AGD-affected fish has occurred in other experimental situations. For example, dorsal aorta catheterisation of AGD-affected fish is difficult if not impossible to achieve on fish heavily (90 % of filaments were affected with AGD-associated lesions) infected with *Neoparamoeba* spp. (M. Leef personal communication). Therefore the study reported in chapter 2 proceeded until 11 days post-exposure (DPE) when fish had a moderate level of infection (43 % of filaments were affected with AGD-associated lesions). If the infection proceeded longer more leucocytes may have been present in the gill and ROS might have been detected.

Occasionally, an individual fish GC suspension would produce a significant respiratory burst response to PMA compared to the unstimulated cells (data not shown). These isolated incidences suggest that GCs were stimulated during isolation and are consistent with that observed when rainbow trout GCs were isolated (Lin *et al.* 1999). Alternatively, GCs may have been pre-stimulated within the fish itself or leucocytic cell populations in the gills of individual fish are significantly variable. GCs did not adhere to plastic microtitre plates and this is indicative of activation given CpG-oligodeoxynucleotides (CpG) activated

mammalian leucocytes are not plastic adherent. CpG activated cells produced an unknown heat-labile factor that prevented their adherence (Macfarlane & Manzel, 1999).

Chapter 2 reports the chemotactic migration of anterior kidney cells but not of GCs. The lack of cellular locomotion by GCs may be explained by the chemoattractant that was used. GCs isolated from rainbow trout were capable of directional migration, however it was concentration dependent and the stimulant used was rainbow trout serum (Lin *et al.* 1999) rather than fetal calf serum (FCS) described here. Furthermore 10-fold more GCs than anterior kidney cells were required suggesting that there were substantially less cells with chemotactic capability in the suspension or fewer cells were receptive to the chemoattractant than in anterior kidney suspensions (Lin *et al.* 1999). Mucus aggregates in the GC suspension may have prevented cells from migrating during the assay. Although it is suggested that it is more probable that leucocytes were not present in high numbers in the GC suspension or were incapable of chemotaxis.

The temporal and spatial distribution of Ig bearing cells in the gill of AGD-affected fish compared to AGD-unaffected fish remained constant up to trial termination (32 DPE) (chapter 3). Processing of *Neoparamoeba* spp. antigens systemically (for example in the anterior kidney or spleen) may result in circulating antibodies (Findlay, Helders, Munday & Gurney 1995; Akhlaghi, Munday, Rough & Whittington 1996; Findlay & Munday 1998; Gross, Carson & Nowak 2004; Vincent, Morrison & Nowak 2006) without the presence of mucosal antibodies (Howard 2001). Furthermore, the numbers of Ig bearing cells

in the gill of AGD-affected fish is the same as un-affected fish (chapter 3) and this may be indicative of a lack of antigen-specific mucosal antibodies. Recent evidence indicates that antibodies isolated from AGD-affected fish bind to carbohydrate epitope (s) associated with *Neoparamoeba* spp. (B. Vincent personal communication). Production of these circulating antibodies might occur via T-cell independent mechanisms as occurs during the production of carbohydrate specific antibodies in mammals (Kobrynski, Sousa, Nahmias & Lee 2005).

The paucity of Ig bearing cells seen in the gills (chapter 3) is consistent with the low concentration of mucus IgM and undetectable levels of anti-*Neoparamoeba pemaquidensis* antibodies in gill mucus of AGD-affected fish (Howard & Carson 1995; Howard 2001). Systemic and mucosal antibodies to specific antigens may not be secreted concurrently and therefore the presence of one does not necessarily preclude the presence of the other. For example, specific antibodies against *Flavobacterium branchiophilum* have been found in the gill mucus of bacterial gill disease-affected brook trout (*Salvelinus fontinalis*) but not in the corresponding serum, indicating the production of specific inducible gill antibody production (Lumsden, Ostland, Byrne & Ferguson 1993). Cutaneous mucus total IgM has been identified by Western blot in samples from AGD-affected Atlantic salmon however no anti-*Neoparamoeba* spp. antibodies were identified (Vincent *et al.* 2006). The results of chapter 3 together with the inability to detect specific mucus anti-*Neoparamoeba* spp. antibodies (Howard & Carson 1995; Howard 2001; Vincent *et al.* 2006) indicate that AGD-affected fish do not produce mucosal antibodies against *Neoparamoeba* spp. either at the site of infection or in

cutaneous mucus. Therefore humoral mechanisms may not provide protection against disease as antibodies will not interact with the pathogen.

There was no evidence of Ig bound to *Neoparamoeba* spp. *in situ* when AGD-affected gill sections were probed for Ig (chapter 3). Whilst this was not specifically investigated in the study, antibodies attached to the surface of *Neoparamoeba* spp. may have been detected by the anti-Atlantic salmon Ig antibody. Antibody binding to the cell surface may have stimulated the classical complement. If no binding occurred, production of C5a, a potent chemoattractant for teleost leucocytes, would not occur (Boshra, Peters, Li & Sunyer 2004). The presence of C5a in association with *Neoparamoeba* spp. may result in significant numbers of leucocytes migrating to the gill. However, anti-*Neoparamoeba* spp. antibodies may not have been detected on the parasites if the concentration of Ig was below detectable limit, Ig was internalised by the parasite or the parasites bound by Ig were no longer attached to the gill.

Anterior kidney immune response to infection

The *ex vivo* production of ROS, locomotion and phagocytic abilities of anterior kidney cells isolated from AGD-affected fish were measured thrice during this thesis (chapters 2, 5 & 6). Interestingly there was variation in the immune response of the fish in all three studies and this was probably related to the different experimental designs (Table 8-1). These results indicate that it is important to standardise *Neoparamoeba* spp. infection as the intensity and duration of the disease may have a significant impact on the immunological and probably physiological response of the fish. There was significant daily fluctuation in the activity of anterior kidney cells isolated from AGD-unaffected

and affected fish over time (chapter 2 & 5). Therefore the leucocytes from AGD-affected and unaffected fish were compared only on the same day and not between days (chapter 2 & 5). However, the study reported in chapter 6 did not have uninfected controls and therefore results were compared within treatment groups and over time.

There was a difference in the infection intensities as adjudged by the proportion of AGD-affected gill filaments (Table 8-1). The phagocytosis rate of *Bicotylophora trachinoti* infected pompano (*Trachinotus marginatus*) was directly proportional to the number of infesting parasites (Chaves, Luvizzotto-Santos, Sampaio, Bianchini & Martinez 2006) and the variability in *Neoparamoeba* spp. infection may have influenced data presented here.

There was an overall trend for leucocytes isolated from AGD-affected fish to have suppressed or equal functions compared to those from AGD-unaffected fish (Table 8-1). The exception being the phagocytic index (6 and 11 DPE) and rate (4 DPE) of anterior kidney cells from AGD-affected fish being elevated occasionally, and the increased chemotactic migration of anterior kidney cells from AGD-affected fish (chapter 5). Together it appears that there is not significant anterior kidney mediated response to AGD. This may be due to the skewing of the immune response towards humoral immunity, hence the antibody production and/or the immunosuppressive effects of *Neoparamoeba* spp..

Table 8-1 Comparison of reactive oxygen species (ROS) production, locomotion and phagocytic ability by anterior kidney cells isolated from Atlantic salmon during 3 separate studies (chapter 2, 5 & 6). Infected = fish exposed to *Neoparamoeba* spp., control = AGD-unexposed fish, DPE = days post *Neoparamoeba* spp.-exposure, FW bathed/SW maintained = fish that were infected with *Neoparamoeba* spp., FW bathed and then maintained in sea water for 6 weeks prior to re-infection. Suppressed, elevated, equal indicates comparison between infected fish and unaffected fish (where applicable) on last sample day.

	Chapter 2	Chapter 5	Chapter 6
Infectious inoculate (cells L^{-1})	400	3300	3300
Neoparamoeba spp. isolation method	Morrison, Crosbie & Nowak (2004)	Zilberg, Gross & Munday (2001)	Zilberg, Gross & Munday (2001)
Infection intensity (%)[Ψ]			
Infected	43	24[¤]	74
Control	0	0	N/A
Tank volume (L)	500	3000	5000
Sample day (DPE)	0, 7, 11	0, 1, 4, 6, 8, 11	0, 19, 26
ROS Production			
PMA stimulated	Equal	Suppressed	Suppressed[◊]
Resting (unstimulated)	Equal	Suppressed	Equal[◊]
Chemotaxis			
Migration ratio	Equal	Elevated	N/A
Phagocytosis			
Phagocytic index	Equal	Equal	Suppressed[◊]
Phagocytic rate	Equal	Elevated	Equal[◊]

[Ψ] Proportion of gill filaments affected with AGD-associated lesions on the last day of the trial
[¤] M. Powell, personal communication
[◊] Results were compared between SW maintained fish 19 and 26 DPE as no unaffected fish were present in that study

Anterior kidney leucocytes of AGD-affected fish at times had decreased ability to produce ROS (chapter 5 & 6). High cortisol levels are an indicator of stress and can cause immunosuppression in fish (Pickering, Pottinger & Carragher 1989; Houghton & Matthews 1990) which for example may manifest as decreased macrophage function (Mustafa, MacWilliams, Fernandez, Matchett, Conboy & Burka 2000). The concentrations of cortisol in the serum of *Neoparamoeba* spp. exposed and unexposed fish (data from a study run concurrently with chapter 5) 0 and 11 DPE were not significantly different ($P > 0.05$) and ranged from 25 - 40 $ng.ml^{-1}$ (M. Powell personal communication). Other unpublished work also indicates that cortisol levels are not elevated in farm-reared AGD-affected

Atlantic salmon (Fazioli 2005). Therefore it appears that changes to immunological responses such as suppressed phagocyte function in AGD-affected fish are not stress mediated. Anterior kidney immune cell functions may be affected by the physiological response to AGD. Hypoxia, hypercapnia and acidosis have been found to cause suppressions in phagocyte function in fish (Angelidis, Baudin-Laurencin, Quentel & Youinou 1987; Boleza, Burnett & Burnett 2001). As AGD is a condition resulting in an altered gill epithelium, it is expected that blood hypoxia may occur. However, AGD-affected fish are not known to be affected by hypoxia (Powell, Fisk & Nowak 2000; Fisk, Powell & Nowak 2002; Powell & Nowak 2003; Leef, Harris & Powell 2005), although respiratory acidosis has been reported (Powell *et al.* 2000; Powell & Nowak 2003; Leef *et al.* 2005) and may play a role in suppressing phagocyte function during AGD.

A variable, but generally elevated phagocytic rate of leucocytes isolated from AGD affected fish anterior kidney was identified in chapter 5, in contrast a generally suppressed phagocytic rate was identified in chapter 6 and in chapter 2 it remained equal. Whilst the phagocytic rate tended to be elevated, the phagocytic index remained comparable on most days. This indicated that the leucocytes were not able to engulf more yeasts but more cells were phagocytosing. Whilst it is not expected that leucocytes isolated from fish would be capable of engulfing *Neoparamoeba* spp. due to the size of the parasite, analysis of phagocytic ability may reflect the overall immune response of the fish and therefore yeast was used as the model target particle for the studies reported in this thesis. It should however be noted that studies of phagocytic ability are

limited by the target particle and do not necessarily reflect the *in vivo* ability of the phagocytes to recognise and engulf other particles. For example the ability of mammalian phagocytes to engulf *Candida albicans* did not always correspond with the ability of neutrophils or macrophages to engulf bacteria. This was due to different cell surface receptors involved in the phagocytic process (Wiener 2003). Moreover Atlantic salmon could phagocytose more *Loma salmonae* than chinook salmon, but equal numbers of yeast cells (Shaw, Kent & Adamson 2001). Thus, the ability to phagocytose one particle type does not necessarily correlate with the ability to phagocytose another. In addition, individual variation, day to day variation amongst samples and infection intensities may also influence phagocytic ability.

After eight DPE, the ability of un-stimulated anterior kidney cells isolated from AGD-affected fish to produce ROS was significantly suppressed compared to cells from AGD-unaffected fish (chapter 5). However, PMA-stimulated cells produced equal amounts of ROS, thus resulting in a significantly increased stimulation index of AGD-affected fish compared to unaffected (chapter 5). This priming response may be a result of up-regulated expression of IL-1β and/or other cytokines in the anterior kidney during *Neoparamoeba* spp. infection. Mammalian IL-1β primes leucocytes for increased ROS production (Niwa, Ozaki, Kanoh, Akamatsu & Kurisaka 1996). However, IL-1β expression in the anterior kidney of both rainbow trout and Atlantic salmon has not been reported to be up-regulated during AGD (Bridle, Morrison, Cupit Cunningham & Nowak 2006a; Bridle, Morrison & Nowak 2006b).

The ability of unstimulated and PMA-stimulated anterior kidney cells isolated from AGD-affected fish to produce ROS was suppressed 11 DPE compared to cells from AGD-unaffected fish (chapter 5). This indicates that cells may have been desensitised after long-term exposure to *Neoparamoeba* spp.. $p40^{phox}$, an essential component for ROS production in mammals (De Coursey & Ligeti 2005) is down-regulated in the gills of AGD-affected fish (Morrison, Cooper, Koop, Rise, Bridle, Adams & Nowak 2006). Whilst $p40^{phox}$ has not been assessed in the anterior kidney of AGD-affected fish, if it were suppressed this may affect ROS production (Ellson, Davidson, Ferguson, O'Connor, Stephens & Hawkins 2006), although it should be noted that whilst the presence of $p40^{phox}$ in fish has been identified (Inoue, Suenaga, Yoshiura, Moritomo, Ototake & Nakanishi 2004) its functional role is yet to be determined.

Host-parasite interactions in vitro

Plasma from AGD-affected and unaffected Atlantic salmon affected the viability of *Neoparamoeba* spp. *in vitro*. However, the effect may be due to the osmolality of the plasma and not anti-parasitic factors as the osmolality control (L15) also significantly affected *Neoparamoeba* spp. viability (chapter 7). *Neoparamoeba* spp. incubated in plasma had greater viability than those incubated in the L15 perhaps due to a buffering effect by the plasma proteins. Furthermore, heat-inactivated plasma affected viability equally as untreated plasma indicating that complement probably did not play a role in decreased viability. There was no demonstration of complement activity in the untreated plasma samples used in chapter 7 however immediate freezing of the plasma sample at -80°C and no repeated freeze-thawing processes would presumably maintain complement integrity. Also, AGD-affected fish may not have had circulating antibodies

present in the serum at the time of sampling which would be essential for classical complement mediated lysis (Boshra, Li & Sunyer 2006).

There was no effect of cutaneous mucus isolated from AGD-affected and unaffected Atlantic salmon on *Neoparamoeba* spp. viability *in vitro* (chapter 7). Mucus from healthy or diseases fish can often kill parasites despite the fact that the parasite colonises the fish skin or gill (Noga & Levy 1995; Harris, Soleng & Bakke 1998). Histone-like proteins (HLPs) have been isolated from the mucus and epidermis of fish and purified forms can have lethal effects on the protozoan ectoparasite *Amyloodinium ocellatum* (Noga, Fan & Silphaduang 2002). HLPs may only function in protecting fish against very aggressive ectoparasites such as *Amyloodinium ocellatum* (Noga *et al.* 2002) which penetrate the skin and gill epithelial cells, thereby coming into close contact with the HLPs (Noga *et al.* 2002). It was surmised that HLPs would be less effective against parasites such as *Trichodina* and *Chilodonella* that reside within the gill mucus layer and on the surface of the epithelial cells (Noga *et al.* 2002). *In vivo* mucus may not have an effect on *Neoparamoeba* spp. because the parasite generally resides on the surface of the gill where the concentrations of humoral immune components such as HLPs may be too dilute to be effective.

Salmonid anterior kidney cells incubated with sonicated *Neoparamoeba* spp. produce a significantly greater amount of ROS when stimulated with PMA compared to cells not pre-incubated with *Neoparamoeba* spp. yet still stimulated

with PMA (chapter 4). This *in vitro* priming response may be a direct result of *Neoparamoeba* spp. actions on anterior kidney cell ROS receptors or down-stream effects such as cytokine production by stimulated anterior kidney cells. The exposure time used during the study (12h) was such that production of IL-1β, TNF-α or other cytokines may have caused cytokine-induced priming of the anterior kidney cells. *In vitro* exposure of carp (*Cyprinus carpio*) peripheral blood leucocytes to a protein produced by *Aphanomyces piscicida* resulted in increased expression of IL-1β mRNA 4h after stimulation (Kurata & Hatai 2002) and *Trypanoplasma borreli* induced the expression of TNF-α mRNA by carp anterior kidney cells when incubated together *in vitro* (Saeij, Stet, de Vries, van Muiswinkel & Wiegertjes 2003). Both of these studies looked only at mRNA expression and not translation therefore protein concentrations were unknown. The significance of the production of these cytokines is that in mammals these proteins prime neutrophils for enhanced ROS production (Niwa *et al.* 1996). In addition it has been reported that intraperitoneal injection of the predicted TNF-α peptide into seabream (*Sparus aurata*) resulted in priming of the respiratory burst produced by anterior kidney cells (Garcia-Castillo, Chaves-Pozo, Olivares, Pelegrin, Mesequer & Mulero 2004). Therefore, induction of TNF-α and IL-1β expression by anterior kidney cells exposed *in vitro* to *Neoparamoeba* spp. may result in *in vitro* priming of the respiratory burst response and an increased ability to produce PMA stimulated ROS following parasite exposure. Hypothetical mechanisms by which *in vitro* and *in vivo* exposure of leucocytes to *Neoparamoeba* spp. may alter the production of ROS is shown (Figure 8-1).

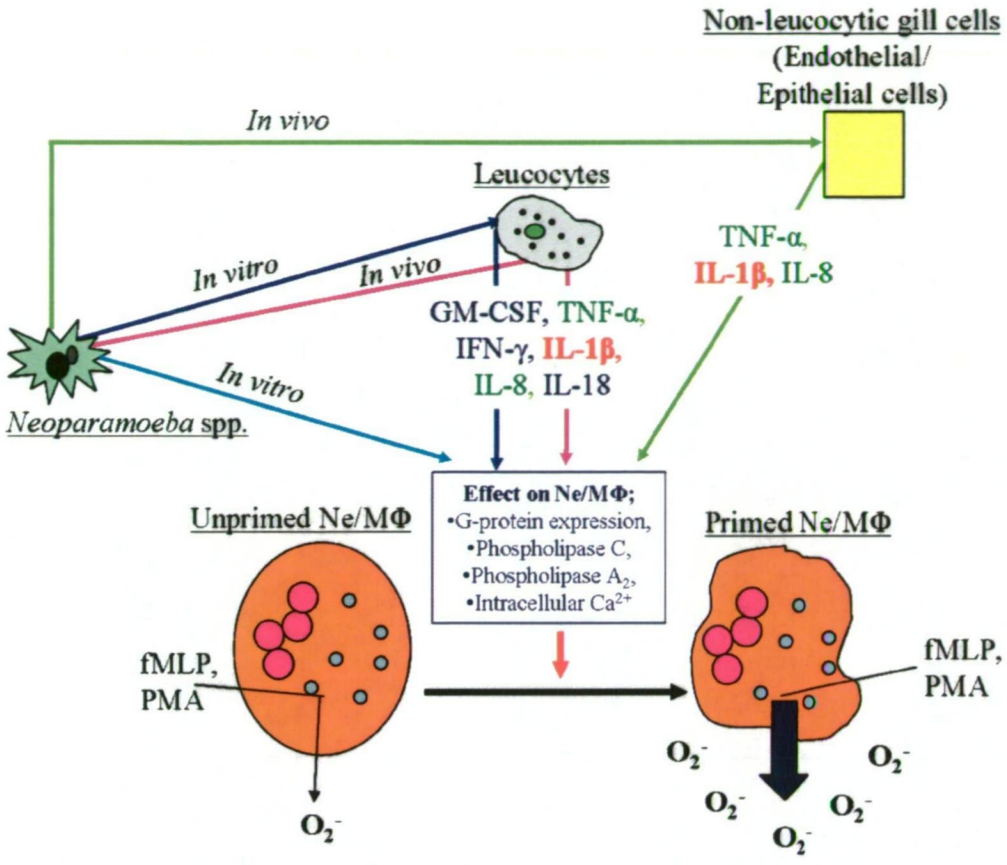

Figure 8-1 Hypothetical process of phagocyte (neutrophil (Ne)/macrophage (MΦ)) priming in fish caused by *Neoparamoeba* spp. exposure *in vivo* or *in vitro*. Exposure to the parasite may result in the production of cytokines or other inflammatory mediators which act on cytosolic components (blue square) and prime the cells for enhanced production of superoxide anion (O_2^-) following subsequent stimulation with activators such as phorbol myristate acetate (PMA) or formylmethionyl-leucyl-phenylalanine (fMLP). Potential priming agents include; tumor necrosis factor α (TNF-α), granulocyte/macrophage colony stimulating factor (GM-CSF), interleukin-1 (IL-1), interleukin-8 (IL-8), interleukin-18 (IL-18), interferon-γ (IFN-γ) and lipopolysaccharide (LPS). Red cytokine = up-regulated expression during AGD, green cytokine = unchanged expression during AGD, blue = expression yet to be determined during AGD, arrows of the same colour indicates pathway of activation (Figure constructed from results reported in this thesis and R. Morrison personal communication; Condliffe, Kitchen & Chilvers 1998; Neumann, Stafford & Belosevic 2000; Fujiki, Gauley, Bols & Dixon 2003; Huising, Stet, Savelkoul & Verburg-van Kemenade 2004; Sigh, Lindenstrom & Buchmann 2004; Elbim, Guichard, Dang, Flay, Pedruzzi, Demur, Pouzet, El Benna & Gougerot-Pocidalo 2005; Bridle *et al.* 2006a; Bridle *et al.* 2006b; McBeath, Snow, Secombes, Ellis & Collet 2006).

Sonicated *Neoparamoeba* spp. did not affect the ability of anterior kidney cells to produce a respiratory burst after 12h exposure *in vitro* (chapter 4). Should *Neoparamoeba* spp. secrete or excrete substances that are cytotoxic, this may have affected the production of ROS by anterior kidney cells. Incubation of *Neoparamoeba* spp. with gill epithelial cells results in cytopathic effects (CPE) and cytolysis of cell monolayers (Butler & Nowak 2004; Lee, Van Es, Walsh, Rainnie, Donay, Summerfield & Cawthorn 2006). Anterior kidney cells were incubated with *Neoparamoeba* spp. for 12h at which time ROS production was assessed (chapter 4). *N. pemaquidensis* were cytopathic for rainbow trout gill and skin cell-lines within 24 - 48h, whilst it required 4 days before CPEs were observed in other cell lines (Lee *et al.* 2006) and cytolysis of the Atlantic salmon GC line was not complete until 5 days post-incubation (Butler & Nowak 2004). If the anterior kidney cells (chapter 4) were incubated with the sonicated *Neoparamoeba* spp. for greater than 12h CPEs may have occurred and destruction of the anterior kidney cells would have affected their ability to produce ROS. However, neither of the previous studies used bacterial controls and it is therefore impossible to determine whether CPEs were the result of *N. pemaquidensis* or associated bacteria (Butler & Nowak 2004; Lee *et al.* 2006).

Sonicated *Neoparamoeba* spp. is not a chemotactic stimulant for anterior kidney cell locomotion *in vitro* (chapter 4). In addition, *in vivo Neoparamoeba* spp. infection does not up-regulate IL-8 mRNA expression in the gill of AGD-affected fish (Bridle *et al.* 2006b). These results suggest that *Neoparamoeba* spp, does not stimulate leucocyte chemotaxis either directly (*in vitro*) or indirectly (*in vivo*) and may indicate the production of immunosuppressive products by *Neoparamoeba*

spp.. The polarised chemotactic response of anterior kidney cells isolated from carp that were naturally or experimentally exposed to the intestinal worm *Bothriocephalus acheilognathi*, was suppressed compared to naïve fish (Nie & Hoole 2000). The authors suggested this may have been due to immunosuppressive products produced by the parasite which were inhibiting anterior kidney cell chemotaxis (Nie & Hoole 2000).

Based upon evidence reported in this thesis and the literature regarding AGD-affected fish immune responses a figure describing the hypothetical interactions that may occur between host immune factors and *Neoparamoeba* spp. has been described (Figure 8-2). The figure shows how *Neoparamoeba* spp. may stimulate production of circulating antibodies by T-cell independent mechanisms and that this results in suppression or no stimulation of cellular immune responses such as ROS or RNI production.

Resistance to AGD

Induction of resistance against AGD has been reported by Findlay *et al* (1995), Findlay & Munday (1998), Vincent *et al* (2006) and in this thesis (chapter 6). All of the studies used different experimental protocols and had contrasting results (Table 8-2). AGD-affected Atlantic salmon maintained long-term in fresh water prior to a secondary exposure were resistant to disease compared to first time infected fish and this was surmised to be due to stimulation of the innate immune response (Findlay *et al.* 1995; Findlay & Munday 1998; Findlay 2001). Fish that were exposed to *Neoparamoeba* spp. and then freshwater bathed for 24h and re-infected were protected from AGD compared to first time infected fish (Vincent

et al. 2006). Specific antibodies were detected in the serum of 50 % of the resistant fish (Vincent *et al.* 2006) although whether the resistance was associated with the antibodies was not assessed and it seems unlikely given only 50 % of resistant fish had parasite-specific antibodies. Fish that were freshwater bathed to resolve a primary infection and maintained in seawater for 6 weeks until they were re-infected with *Neoparamoeba* spp. were less susceptible to AGD compared to fish infected for the first time (chapter 6).

It is apparent from these data that resistance to AGD can be influenced by experimental variables such as infection intensity, diseases duration and method by which resistance is measured (Table 8-2). The duration of the primary infections presented by Findlay *et al* (1995), Findlay & Munday (1998) and Vincent *et al* (2006) were much longer and less intense (no mortalities) than that reported in chapter 6, furthermore temperatures were much lower in the studies reported by Findlay *et al* (1995), Findlay & Munday (1998) and Vincent *et al* (2006) which is consistent with the longer disease duration. Methods of infection and inoculation densities were different with Vincent *et al* (2006) introducing only 500 cells L^{-1} while Findlay *et al* (1995) & Findlay & Munday (1998) infected via co-habitation. Both of these methods may result is slower infection dynamics as was demonstrated by experimental duration (Table 8-2). Exposure time to the parasite without significant challenge (no mortalities) may be an important factor in disease resistance. The variability in disease resistance could suggest that resistance in the laboratory may not always be reproduced in commercial culture conditions.

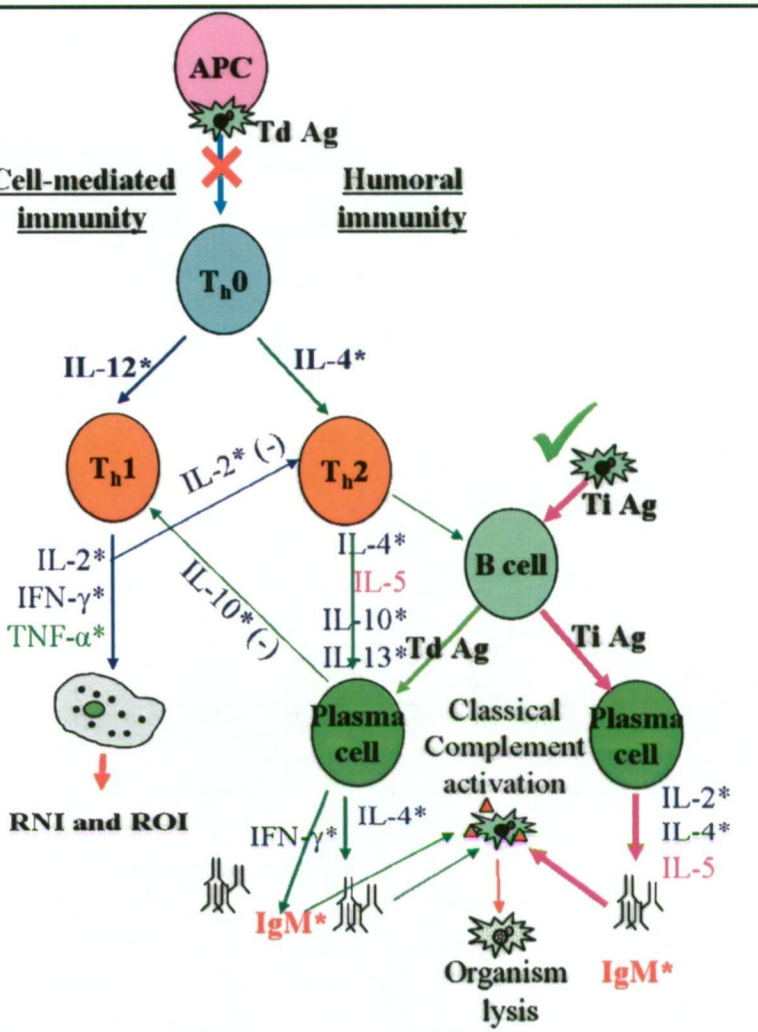

Figure 8-2 Hypothesised immune response of Atlantic salmon during AGD. The function of AKCs isolated from AGD-affected fish may be suppressed during infection (Chapter 5 & 6) and there seems to be little ability of GCs to produce ROS (GCs) (Chapter 2) which suggests that the T_h1 immune response pathway is not stimulated (no T dependent, Td processing). The presence of circulating (low titre) parasite-specific antibodies in the serum of fish suggests the T_h2 immune response is stimulated. Antibodies putatively bind to carbohydrate epitopes, which may therefore indicate T-cell independent (Ti) processing of *Neoparamoeba* spp.. Either T_h2 or T-cell independent immune responses result in suppression of T_h1 immune responses which ultimately results in suppression of ROS and RNI production. Red IgM = presence of parasite specific antibodies during AGD, green cytokine = no change in regulation during AGD, * cytokine present in fish, pink cytokine = not yet identified in fish, blue cytokine = expression not yet determined during AGD, same colour arrows indicate pathway of activation (Figure constructed from results reported in this thesis and R. Morrison personal communication; C. Secombes personal communication; B. Vincent personal communication; Vallejo, Miller & Clem 1992; Kubin, Kamoun & Trinchieri 1994; Laichalk, Danforth & Standiford 1996; Constant & Bottomly 1997; Steinman 2001; Gross *et al.* 2004; Lindenstrom, Secombes & Buchmann 2004; Sigh *et al.* 2004; Bird, Zou, Kono, Sakai, Dijkstra & Secombes 2005; Inoue, Kamota, Ito, Yoshiura, Ototake, Moritomo & Nakanishi 2005; Bridle *et al.* 2006a; Bridle *et al.* 2006b; Huising, van Schijndel, Kruiswijk, Nabuurs, Savelkoul, Flik & Verburg-van Kemenade 2006; McBeath *et al.* 2006; Vincent *et al.* 2006).

Table 8-2 Experimental designs and outcomes of the major studies investigating the induction of AGD resistance in Atlantic salmon. Bold type indicates treatment groups of fish that were significantly protected from disease in each study. FW = fresh water maintained after first infection, SW = sea water maintained after first infection, naïve = fish never exposed to *Neoparamoeba* spp..

	Findlay *et al* (1995)	**Findlay & Munday (1998)**		**Chapter 6**	**Vincent *et al* (2006)**
		Trial 1	Trial 2		
Salinity (‰)	Unknown	Unknown		36 (± 1)	35
Temperature (°C)	14	14		17 (± 0.5)	12 (first exposure) 16 (second exposure
Treatment groups	**FW maintained** FW bathed/SW maintained Naïve	**FW bathed** Naïve	FW maintained ×2 FW bath ×1 FW bath Naive	FW maintained **FW bathed/SW** maintained Naïve	**FW bathed** Naïve
First time exposure (weeks)	4	4		2	4
Freshwater bath duration (h)	None	2		4	24
Resolution time (weeks)	4	4		6	None
Second time exposure (weeks)	4	4		4	5
Assessment of infection severity	Gross gill lesion scores	Gross gill lesion scores		Cumulative mortality Histology	Cumulative mortality Histology
Proportion affected filaments of surviving fish				FW maintained (91 %) FW bathed/SW maintained (74 %) Naïve (68 %)	First exposure (≈88 %) Second exposure (≈90 %)
Infection method	Cohabitation	Cohabitation		Inoculation$^{\Diamond}$ (3300 cells L^{-1})	Inoculation$^{\Phi}$ (500 cells L^{-1})

$^{\Phi}$ *Neoparamoeba* spp. were isolated using the plastic adherence method as per Morrison, Crosbie & Nowak (2004)

$^{\Diamond}$ *Neoparamoeba* spp. were isolated using the gill mucus scrape method as per Zilberg, Gross & Munday (2001)

Fish maintained in sea water after an initial infection had better survival compared to first time infected fish and fresh water maintained fish when they were challenged with *Neoparamoeba* spp. for a second time (chapter 6). However there was no difference in the severity of the AGD (as assessed by histology) between surviving fish and dead fish (chapter 6). Results from chapter 6 correlate with those of Vincent *et al* (2006) in that the gill histopathology of surviving fish was not significantly different between resistant and susceptible fish. As gill histology is the same between resistant and susceptible fish it suggests that resistance may be mediated by physiological adaptation rather than immunologically mediated processes, which might result in resolution of gill pathology. It is interesting to note that Findlay *et al* (1995) and Findlay & Munday (1998) used the number of mucoid patches on the gill of affected fish as a measure of resistance. The fish deemed resistant to AGD had significantly fewer patches than susceptible fish. Providing no other gill condition is present mucoid patches correlate well with histological lesions (Adams, Ellard & Nowak 2004). Therefore assuming that no other gill condition existed in the studies reported by Findlay *et al* (1995) and Findlay & Munday (1998), the histology should have reflected the macroscopic appearance and those resistant fish would have had a lower number of histological lesions, which would have been an example of gill pathology resolution.

Future directions

Induction of resistance against AGD has been reported by Findlay *et al* (1995), Findlay & Munday (1998), Vincent *et al* (2006) and in this thesis (chapter 6) however, the mechanisms by which this occurs are unknown. Future research

should identify the immunological (or other) mechanisms by which this resistance occurs. To do this, resistance should be induced in a group of fish (by similar mechanisms as those reported and discussed in this thesis) and immunological parameters such as those studied in this thesis and others such as cytokine profiles compared between resistant and susceptible fish. Resistance would need to be defined and fish would need to be identified in the population as such.

Incubation of *Neoparamoeba* spp. with anterior kidney cells may stimulate the secretion of cytokines (chapter 4). The development of an *in vitro* model is required to assess the effect that *Neoparamoeba* spp. has on host cells. This model would require freshly isolated *Neoparamoeba* spp. as differences in antigenicity between wild-type and cultured strains have been reported (Villavedra, McCarthy, Jo, Morrison, Crosbie & Raison 2005; Vincent *et al.* 2006). Furthermore, aseptic techniques for parasite isolation would be preferable as bacterial contamination could influence results. An *in vitro* cell model would enable the cytokine profile of anterior kidney cells and GCs when incubated with *Neoparamoeba* spp. to be investigated. This may clarify whether *Neoparamoeba* spp. directly stimulate the production of IL-1β and assist in the identification of other host-parasite interactions. Also, various components of the NADPH-oxidase pathway may be investigated to identify how *Neoparamoeba* spp. primes cells for ROS production. In particular $p47^{phox}$ activity should be examined as partial phosphorylation of this component is often responsible for priming of mammalian neutrophils (Dewas, Dang, Gougerot-Pocidalo & El Benna 2003). The presence of HLPs in Atlantic salmon mucus and epidermal tissue and their effects on *Neoparamoeba* spp. viability should also be investigated as this may

identify potential pathways of *Neoparamoeba* spp. inactivation that are host-mediated.

Development of antibodies specific for Atlantic salmon leucocyte antigens would be a great advantage for future immunohistochemical studies that were to be performed on AGD-affected gills. Such studies could investigate population dynamics of leucocytes in other organs such as the intestine, anterior kidney, spleen and thymus. Turbot infected with the parasite *Enteromyxum scophthalmi* have significant increases in Ig bearing cells in the intestine, spleen and anterior kidney which was associated with initiation of the humoral immune response (Bermudez, Vigliano, Marcaccini, Sitja- Bobadilla, Quiroga & Nieto 2006). Whilst Ig bearing cell numbers do not increase in the gill of AGD-affected fish (chapter 3) antibodies are present in the circulation (Findlay *et al.* 1995; Akhlaghi *et al.* 1996; Howard 2001; Gross *et al.* 2004; Vincent *et al.* 2006) and therefore changes in Ig bearing cell numbers may occur in other lymphoid tissues.

Further research may focus on identifying techniques to assess the *in vitro* function of GCs and to identify how their activity may be enhanced. The inclusion of β-glucans in the diet of Atlantic salmon did not induce significant resistance to disease (Bridle, Carter, Morrison & Nowak 2005), however CpG treated fish were protected from disease compared to untreated fish (Bridle, Butler & Nowak 2003). Dietary inclusion of immunostimulant preparations such as *Candida utilis, Saccaromyces cerevisiae*, Chitosan and FinnStim have been reported to increase innate immune parameters such as ROS production,

phagocytosis and bactericidal activity of leucocytes isolated from rainbow trout anterior kidney (Siwicki, Anderson & Rumsey 1994).

In vivo cytokine profiles other than those already reported could be measured during AGD. For example the expression of members of the CXC chemokine family and members of the CC family of chemokines should be investigated to further elucidate inflammatory responses during AGD. In mammalian disease models the activation of cellular components of the innate immune system during infection with protozoan parasites relies upon the pathogen to stimulate monocytic cells to produce interleukin-12 (IL-12) and TNF-α and interferon-γ (IFN-γ) (Kubin *et al.* 1994; Rossi & Zlotnik 2000). Identification of cytokine expression profiles IFN-γ, IL-12, IL-10, and IL-2 may give an indication of whether *Neoparamoeba* spp. infection elicits a T_h1 or T_h2 (T helper) induced T-cell dependent response of if in fact T-cell independent processing occurs. These results would not only be novel within the context of AGD research but would also provide knowledge towards the understanding of the fish immune system as only recently has a T_h2 cytokine (IL-4) been isolated from fish (C. Secombes, personal communication).

Conclusion

It has been suggested throughout this thesis that AGD-affected Atlantic salmon do not mount an immune response either in the gill or anterior kidney that is adequate for disease resolution. ROS production, chemotaxis and phagocytosis by anterior kidney (chapter 5 & 6) and gill (chapter 2 and 3) cells are not enhanced by infection, nor when the parasite is in direct contact with host immune cells (chapter 4). Furthermore tempo-spatial variation in Ig bearing cells

did not occur during AGD, which indicates that the gill is not a site for production of anti-*Neoparamoeba* spp. antibodies. Relative percent survivals of greater than 60 %, which is often considered a statistically significant level of protection (Amend 1981), have never been achieved in fish putatively described as resistant to AGD. Furthermore resistance has been variable and dependent upon the method by which it was measured. It is suggested that if the fish were to mount a substantial cellular immune response both in the gill or anterior kidney then resolution of the disease may occur and that future research should focus on clarifying the pathways by which *Neoparamoeba* spp. affect host cells.

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Appendices

Appendix 1 – animal ethics reference numbers

Project name	University of Tasmania animal ethics committee approval number
Infections and resistance of Atlantic salmon to AGD	A0006592
Infections and resistance of Atlantic salmon to AGD	A0006592
AGD: host – pathogen interactions	A0006092
Immunological response of Atlantic salmon to AGD	A0007690

Appendix 2 – Papers published by the author.

These l·ŃŭĺǏfŜs have been removed for copyright or proprietary reasons.

Gross, K. A., Morrison, R. N., Butler, R. and Nowak, B. F. 2004), Atlantic salmon, Salmo salar L., previously infected with Neoparamoeba sp. are not resistant to re-infection and have suppressed phagocyte function. Journal of Fish Diseases, 27: 47–56
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